QUANTITATIVE
CLINICAL CHEMISTRY

PREFACE

The present volume, following the plan outlined in the preface to Volume I, endeavors to present methods for the determination of those substances, found in the body and its excreta, which are of importance for clinical medicine, and for the estimation of which suitable quantitative methods are available. It has been the aim of the authors to describe for each substance methods of different types, so that the reader may choose the procedures that best fit his laboratory facilities, personal preferences, and the conditions of the moment. The original plan was to select for each substance a gravimetric, a titrimetric, a colorimetric, and a gasometric procedure, when desirable methods for each were available with, when advisable, macro and micro forms. This plan has been followed with much elasticity. In some cases the popularity of two procedures of the same type has made it desirable to present both. In other cases one type of analysis has been omitted because it offers at present no methods able to compete in convenience or accuracy with current procedures of other kinds. Thus, for urine sugar, both Benedict and Shaffer titrations are given, and no gravimetric analysis. In general, however, the authors have endeavored to adhere to the original plan. It has involved the exercise of a certain amount of arbitrary selection, and many methods have been omitted which doubtless equal those presented.

It appeared desirable that the volume should be something more than an uncritical manual of technique. The practice has therefore been followed of prefacing each chapter with a discussion of the principles on which the methods are based. These discussions usually cover other applicable procedures in addition to those detailed in this volume. It has been hoped that thereby the reader might secure, not only a critical view of the methods described, but also guidance to others in the literature.

It was the intention of the authors to test in their laboratories each method described, and if possible to extend the test to routine analyses. This plan has been but incompletely approximated at a time when it seems undesirable longer to delay publication. However, the methods not personally tested have, it is believed, undergone such usage in other laboratories that confidence may be placed in them.

For calculations, factors are routinely given to reckon determined substances in terms of molecular or equivalent concentrations in both urine

and blood, in grams of substance per liter in urine, and in milligrams per 100 cc. in blood. It would be preferable for simplicity and uniformity to use grams per liter for blood as well as urine, but the milligrams per 100 cc. unit for blood is at present retained in deference to general usage in this country.

The principle, of grouping in one chapter all the procedures described for the determination of a given substance, has been violated with regard to the gasometric methods. The gasometric methods for urea, sugar, etc., instead of being placed in the chapters devoted to these substances, are there indicated only by references to the general gasometric chapter, in which they are detailed. Collecting all the gasometric methods in one part of the volume has seemed desirable because of the frequent cross references to points of technique. Also, for analysts who do not use the gas apparatus, the other chapters are simplified by the omission of gasometric methods. The gasometric methods in Chapter VII differ in some details from the descriptions originally published. Such differences are due to improvements made in these procedures during the interval since their publication.

An appendix, with methods for renal and hepatic function tests by means of phthalein dyes, and for semi-quantitative estimation of bile pigments in blood plasma, has been added. These procedures are not strictly within the scope of quantitative analyses outlined for this volume, but they are required so frequently from the clinical laboratory that their addition has seemed desirable. In the appendix have also been added methods for chlorides in gastric contents, and for several quantitative procedures that were not available when the main text was prepared.

From this edition one entire class of reliable methods has been omitted, those based by Pregl on the micro balance. The omission has been due, not to failure to appreciate these methods, but to the fact that relatively few clinical laboratories have as yet the facilities for their application.

So many colleagues have assisted the authors with advice, criticism and suggestions, that it would be impossible to acknowledge here the entire debt. However, certain obligations are too definite to be overlooked. Prof. A. B. Hastings has supplied in advance of publication a description of the gasometric micro method for lactic acid, and a more complete description than any hitherto published of the method for determining both pH and CO_2 on 0.1 cc. blood samples. Prof. P. A. Shaffer has revised the account of his sugar methods. Dr. Walther Goebel has furnished an especially practical modification of the Pregl micro Kjeldahl method. Descriptions in advance of publication have been supplied by Dr. J. Sendroy, Jr., for carbon monoxide determination in air, by Prof. A. T. Shohl for a

phosphate method, and by Dr. A. M. Butler and Dr. Eaton M. MacKay for applications of gravimetric methods for the mineral bases. Dr. Roger S. Hubbard has provided practical revisions of his methods for the determination of blood and urine ketones and blood sulfate. Dr. Alma Hiller and Dr. J. Sendroy Jr. have examined nearly the entire manuscript and made many improvements. Finally the authors' thanks are due to many colleagues for permission to reproduce their published methods.

As in the first volume, the authors are indebted for skilled assistance to Miss Elsie C. Nicholls. Miss Ruth Insull, and Charlotte Peters, who have again carried out much of the bibliographical work and preparation of the text, and to Miss Helen Mandelbaum, who prepared many of the illustrations.

JOHN P. PETERS.
DONALD D. VAN SLYKE.

CONTENTS

CHAPTER I

General Chemical Technique

CHAPTER II

Special Biochemical Technique

CHAPTER III

Analyses of Gas Mixtures

CHAPTER IV

Carbon Dioxide and Oxygen Tensions in Alveolar Air

CHAPTER V

Respiratory Metabolism

CHAPTER VI

Lung Volume

CHAPTER VII

Gasometric Methods for Analysis of Blood and Other Solutions

CHAPTER VIII

SUGAR

CHAPTER IX

LIPOIDS

CHAPTER X

Total and Non-Protein Nitrogen

CHAPTER XI

Urea

CHAPTER XII

Urinary Ammonia

CHAPTER XIII

Amino Acids

CHAPTER XIV

Uric Acid

CHAPTER XV

Creatine and Creatinine

CHAPTER XVI

Lactic Acid

CHAPTER XVII

Acetone, Acetoacetic Acid, and Beta-Hydroxybutyric Acid

CHAPTER XVIII

Total Organic Acids

CHAPTER XIX

Phenols

CHAPTER XX

Hemoglobin and Its Derivatives

CHAPTER XXI

Proteins of Urine, Blood Plasma and Body Fluids

CHAPTER XXII

Blood Volume

CHAPTER XXIII

Total Base

CHAPTER XXIV

Sodium and Potassium

CHAPTER XXV

Calcium

CHAPTER XXVI

Magnesium

CHAPTER XXVII

The pH of Blood and Urine

CHAPTER XXVIII

Bicarbonate

CHAPTER XXIX

Titratable Acid and Acid-Base Excretion in Urine

CHAPTER XXX

Chloride

CHAPTER XXXI

Phosphorus

CHAPTER XXXII

Sulfur

APPENDIX

ILLUSTRATIONS

CHAPTER I

General Chemical Technique

To review the principles of quantitative analytical technique is beyond the scope of this volume, which is intended for readers who are already familiar with the general details of quantitative analysis. There are, however, certain procedures which are peculiar to or have a special significance in biological analysis which are not covered in the usual manuals. These and a few general precautions and points which experience has proved to be worthy of repetition are considered in this and the next chapter.

USE OF THE BALANCE[1]

Balances which are most suitable for use in a biochemical laboratory are of three kinds:

1. A fine analytical balance for gravimetric analyses and for preparing volumetric standard solutions. Such a balance should have a capacity of from 100 to 200 grams with a sensibility of 0.1 mg. when it is fully loaded. Some of the newer balances with devices which obviate use of the smaller weights greatly expedite gravimetric determinations. Among such devices are the "Chainomatic" balance manufactured by Christian Becker, New Rochelle, N. Y., and the Curie air-damped balance, made by the Societé Centrale de Produits Chimiques, 44 Rue des Écoles, Paris. A Sartorius model on the same principle is now on the market. Precision in weighing can be ensured by use of a standard set of weights, of which the accuracy is certified within certain limits by the Bureau of Standards. Equal precision can be more cheaply gained by the use of ordinary weights, of which the corrections have been determined Bureau of Standards. The standard weights may be employed to check other weights which are regularly used.

2. A large balance with a capacity of 5 to 10 kg. and a sensibility of 0.05 gram or less.

3. Trip scales to balance tubes for the centrifuge and to weigh materials for the preparation of solutions that do not have to be extremely accurate.

[1] For a general discussion of the use and standardization of balances the reader is referred to Circular No. 3 of the United States Bureau of Standards, entitled, "Design and Test of Standards of Mass" (4). The principles governing the use of the balance, the use and calibration of volumetric apparatus, and general accuracy in measurement are discussed in Ostwald-Luther's "Physiko-chemische Messungen."

Microbalances can be procured which permit weighing small amounts of material with an error of less than 0.01 mg. Such microbalances have been used for microgravimetric determinations in clinical medicine. These microgravimetric techniques, which Pregl has especially developed, have not been described in this volume, because at present relatively few clinical laboratories possess such balances or analysts trained in their use.

A balance for accurate quantitative work must be perfectly level as indicated by the plumb-bob or spirit level with which it should be equipped. It may be leveled by adjusting the thumb screws beneath the right and left front corners.

All objects must be at room temperature when weighed. Warm or cold objects produce currents of air in the balance case which interfere with accurate weighing.

Crucibles should be cooled in a desiccator if the precipitates which they contain tend to take up water from the air. If the crucible gains weight appreciably during the process of weighing, it should be reheated, cooled in a desiccator, and weighed again very quickly. The weights to balance the crucible approximately are, in this case, placed on the pan before the crucible is removed from the desiccator. A platinum crucible should be left in the desiccator ten to fifteen minutes, a procelain crucible twenty to twenty-five minutes, before it is weighed.

When, as in the case of barium sulfate, the precipitate is not hygroscopic, a desiccator should not be used. Accurate results are more readily obtained if the crucible, both before and after the precipitate is in it, can be cooled in the open. The time required for cooling is only about half as long in the open as in a desiccator.

When the balance is not in use the case must never be left open or with the beam unsupported, and the rider must always be removed from the beam.

The true zero point of the balance must be determined from time to time by noting the point on the scale at which the rider must be placed in order to make the empty pans balance.

Weighing by swings. **In making a weighing after the rider has been so located that the pans balance within a milligram, time can be saved by estimating tenths of a milligram by the method of counting the swings of the pointer. One first determines the number of degrees on the pointer scale, by which the difference between right and left swings is changed by shifting the rider 1 mg.; e.g., with a 10-gram weight in each pan, the rider is so placed that the point of balance is at exactly zero. The rider is then shifted 1 mg. to the right, the pans are set in motion, and the**

excursions of the pointer to right and left are noted on the scale. If they are: left 6.2, right 1.1, left 6.0, the displacement to the left is the mean of the two left swings $\frac{6.2 + 6.0}{2} = 6.1$, minus the right swing, 1.1, or 5.0 degrees to the left. This difference indicates the sensibility of the balance.

In subsequent weighings one can then assume that each 0.5 degree of difference between right and left swings corresponds to 0.1 mg. difference in the loads on the pans.

Each balance has on the pointer shaft a weight which can be moved up and down, to regulate the sensibility. It is convenient to adjust this weight so that, with loads of the magnitude most frequently weighed, the difference between right and left swings caused by 1 mg. will be about 5 degrees on the pointer scale.

The sensibility of the balance varies somewhat with the load, and should be determined with loads of 1, 10, 20, 50, and 100 grams.

USE AND CALIBRATION OF VOLUMETRIC MEASURING APPARATUS

Meniscus readings. With colorless solutions the bottom of the meniscus is read; with colored solutions, such as permanganate, the reading is made at the top of the column of fluid. To avoid errors of parallax the reading must be made with the eye at the level of the top of the column of fluid. To assist in locating the eye at the level of the mark which is read, the chief calibration marks should completely encircle a burette and other graduations should extend half-way round it.

Solutions, when they are measured, should be within 10° of the standard temperature engraved on the volumetric apparatus.

When not in use burettes should be inverted or protected from dust by caps.

Burettes and pipettes must be free from grease or the solution will not moisten the surface of the glass evenly and will collect in droplets. The glass may be cleaned with acid cleaning solution (1 gram of potassium bichromate and 100 cc. of concentrated sulfuric acid). Burettes are allowed to stand for some hours filled with the mixture. Pipettes may be immersed in the mixture in cylinders. They are then washed in water and finally rinsed with distilled water. Burettes may be cleaned quickly by scrubbing with a soapy burette brush.

A minimum of lubricant should be used on *burette stop-cocks.* For burettes which are used for dilute alkali a vaseline lubricant containing

some rubber (see p. 47) is used. For alkali as strong as 0.1 N, burettes with silver stop-cocks are a great convenience because they do not "freeze." When an alkali solution stronger than 0.1 N is used in a stop-cock burette, the burette must be cleaned as soon as the day's analyses are completed. Otherwise the cock is likely to be ruined.

When maximum accuracy is not necessary, the trouble encountered in measuring alkali solutions through glass cocks may be obviated by the use of burettes provided with pinch-cocks made by spring clamps and rubber tubes.

Both burettes and pipettes are calibrated to deliver water, and will not deliver with equal accuracy fluids that differ greatly from water in viscosity, cohesion, or surface tension.

All apparatus for accurate work must be calibrated. Except for that checked by the United States Bureau of Standards commercial apparatus can rarely be assumed to be entirely reliable; errors exceeding 1 per cent are not uncommon. The limit of error tolerated for volumetric apparatus in general is $\pm$0.1 per cent or 1 part in 1000. This accuracy can, however, be attained only if the apparatus is employed for measuring under the same conditions under which it has been calibrated. These include temperature, cleanliness, manner of reading the meniscus, and rate of outflow in apparatus calibrated for delivery.

When water is run out of a burette or pipette a film of the liquid remains adherent to the walls. The more rapid the rate at which the vessel is emptied the more liquid remains on the walls. *In order to deliver amounts constant to within 1 part per 1000 it is necessary that the surface of the liquid descend in the vessel at a sufficiently slow and steady rate, so that the residual film shall be slight and constant.*

A minimum film is left when the rate of descent is slower than 0.5 cm. per second, but rates as high as 1 cm. per second may be consistent with constant delivery, if delivery is always at the same rate; e.g., a 50 cc. burette, with the meniscus falling at the rate of 0.8 mm. per second, delivered regularly 24.95 cc. at the 25 cc. mark. When the rate of fall was retarded to 0.5 cm. per second, the delivery was 25.00 cc., and this was not changed by further retardation to 0.3 or 0.1 cm. per second.

For vessels, such as flasks and sometimes pipettes, calibrated, not to deliver, but to contain a definite volume of liquid, rate of emptying is of course without influence.

Flasks

Construction. Flasks should be constructed with necks of such a caliber that a volume change of 1 part per 1000 causes a noticeable alteration in the

TABLE 1

INNER DIAMETERS OF NECKS OF VOLUMETRIC FLASKS

	CAPACITY OF FLASK							
	2000 cc.	1000 cc.	500 cc.	250 cc.	200 cc.	100 cc.	50 cc.	25 cc.
Maximum diameter in millimeters....	25	20	18	15	13	12	10	8
Minimum diameter in millimeters....	18	14	12	10	9	8	6	6

TABLE 2

APPARENT WEIGHTS AND VOLUMES OF WATER AND MERCURY WEIGHED IN AIR

For use in calibration of volumetric apparatus

TEMPERATURE	WEIGHT OF 1 CC. OF WATER	VOLUME OF 1 GRAM OF WATER	WEIGHT OF 1 CC. OF MERCURY	VOLUME OF 1 GRAM OF MERCURY
°C.	*gram*	*cc.*	*grams*	*cc.*
15	0.9979	1.0021	13.558	0.07376
16	78	22	55	77
17	77	23	53	78
18	75	25	51	79
19	73	27	49	81
20	72	28	47	82
21	70	30	45	83
22	68	32	43	84
23	66	34	41	85
24	64	36	39	86
25	61	39	37	87
26	59	41	34	89
27	56	44	32	90
28	54	46	30	91
29	51	49	28	92
30	48	52	26	93

The figures in this table are based on the weights of water and mercury per cubic centimeter which must be weighed under ordinary conditions into a glass vessel, in order to indicate the mark to which the vessel when at 20° must be filled in order to contain the desired volume of liquid. The figures do not therefore indicate the precise densities and volume weights of water and mercury, but represent these values corrected for the buoyant effect of air upon the fluid weighed and upon the brass weights used to balance it; corrections are also included for the coefficient of expansion of the glass vessel, when the weighing is not done at 20°. The cubic coefficient of expansion of glass increases the capacity of a vessel by only about 1/40,000 for 1° temperature rise, and hence is about one-fourth as important as the expansion of water in volumetric measurements. The figures are from Landolt and Börnstein's "Tabellen."

height of the meniscus. The United States Bureau of Standards (11) prescribes for flasks of various sizes at the point of calibration internal diameters within the limits shown in table 1. The calibration mark must be at least 1 cm. above the point at which the neck of the flask begins to expand into the body and must extend completely around the neck in a horizontal plane.

Calibration. Flasks are calibrated by weighing into them the amounts of water, calculated from the second column of table 2, which are necessary to make the desired volumes at the temperature of calibration. The water should be weighed to 1 part per 1000 i.e., the water held by a 10 cc. flask must be weighed to 0.010 gram, but a 1-liter flask is sufficiently accurate if within 1 gram of the proper weight.

The flask, clean and dry, is weighed. The weight filled at the given temperature is then calculated, and water is introduced to bring the flask to this weight. There must be no globules of water adhering to the neck above the graduation mark when the water is weighed. If the mark on the flask is incorrect a new mark is made at the correct point and later etched as described below. The mark can be made on the glass with a wax pencil sharpened to a chisel edge, or on a strip of gummed paper attached to the neck of the flask. The paper is cut off at the mark before the flask is etched. Another method is to cover the neck of the flask with a thin layer of paraffin, as described below under "etching glass." The correct mark is made through the paraffin. This procedure has the advantage that by it the flask is prepared to have the mark etched in with hydrofluoric acid.

If a balance large enough and sufficiently accurate to calibrate large flasks is not available, these can be calibrated by repeated delivery of water from a calibrated pipette.

Burettes

Construction. The calibration marks of burettes should, for convenient and accurate reading, be about 1 mm. apart. The inner diameter of the burette should be so proportioned to the total capacity that an error of 0.5 mm. in the location or reading of a mark causes an error in volume reading approximating the limit of error permitted for the burette. Table 3 gives for burettes of different size the limits of error in calibration permitted by the Bureau of Standards, the finest subdivisions that should be represented on the scales, and the internal diameters that meet the above requirement.

Furthermore, the United States Bureau of Standards (11) specifies that "The rate of outflow of burettes and measuring pipettes must be restricted by the size of the tip, and for any graduated interval the time of free outflow

must not be more than three minutes nor less than the following (in table 4) for the respective lengths."

Calibration. A burette is calibrated by allowing it to deliver distilled water, 2 cc. or less at a time, into a bottle, and weighing the water. The thoroughly clean burette is first filled with water to a point somewhat above the zero mark. The water is then run out slowly until the meniscus falls to the zero mark. One then waits a minute to insure complete drainage. If the meniscus rises enough water is run out to bring it

TABLE 3

SPECIFICATIONS FOR BURETTES

TOTAL CAPACITY OF BURETTE	LIMIT OF ERRORS PERMITTED BY BUREAU OF STANDARDS (11) FOR TOTAL OR PARTIAL CAPACITY	FINEST SUBDIVISION	INNER DIAMETER
cc.	*cc.*	*cc.*	*mm.*
5	0.01	0.025	5.0 ±0.2
10	0.02	0.05	7.0 ±0.2
25	0.03	0.05	8.7 ±0.3
50	0.04	0.10	11.2 ±0.4

TABLE 4

DELIVERY TIMES FOR BURETTES OF DIFFERENT LENGTHS

LENGTH GRADUATED	TIME OF OUTFLOW	LENGTH GRADUATED	TIME OF OUTFLOW
cm.	*seconds*	*cm.*	*seconds*
65	140	35	60
60	120	20	50
55	105	25	40
50	90	20	35
45	80	15	30
40	70		

again to the zero point. Excess liquid adherent to the tip of the burette is removed. The water is then run in portions into a tared weighing bottle, which contains a layer of paraffin oil a few millimeters thick to prevent evaporation of the water. The oil obviates the necessity of using a cover on the bottle, and the danger of loss by evaporation between weighings that is present when a covered bottle is opened to receive the successive portions of water. The size of the portions of water weighed varies with that of the burette; for 50 and 25 cc. burettes 2 cc. portions are weighed; for smaller ones portions equal to

0.1 the total capacity. After each portion is delivered the tip of the burette is touched to the surface of the paraffin oil in the weighing bottle to detach adherent water.

The temperature of the water is noted, and the grams of water weighed are multiplied by the volume of 1 gram at the observed temperature (third column of table 2) in order to calculate the actual volumes delivered. The figures in table 5, obtained in calibrating the first 10 cc. of a burette, serve as an example.

At least two complete sets of weighings which check should be made in calibrating a burette. It is wise to make occasional observations at intermediate points (fractions of cubic centimeter) to test the evenness of subdivisions.

At each mark the correction is calculated as:

Correction = (actual volume) − (marked volume)

TABLE 5

EXAMPLE OF RESULTS OBTAINED IN CALIBRATING A BURETTE

BURETTE READING	WEIGHT OF WATER DELIVERED AT 22°	VOLUME OF WATER DELIVERED (WEIGHT × 1.0032)	BURETTE CORRECTION
cc.	*gm.*	*cc.*	*cc.*
2	2.000	2.006	+0.01
4	4.002	4.015	+0.02
6	6.009	6.028	+0.03
8	8.020	8.046	+0.05
10	10.020	10.050	+0.05

The + signs indicate that each correction is to be *added* to the volume indicated by the burette scale, in order to obtain the actual volume delivered. Negative corrections would be indicated by minus signs.

Burettes with a capacity of 5 cc. or less may be made to deliver, under certain conditions, quite as accurately as 50 cc. burettes: that is, with an error not exceeding 1 part in 1000 of the total capacity. The construction and calibration of such burettes is described below under the heading "microburettes."

Pipettes

Construction. The uppermost graduation on a pipette should be far enough from the upper end to minimize danger of sucking fluid into the mouth. In transfer pipettes the stem below the bulb should be long enough to permit convenient delivery into a flask or other container.

Transfer pipettes are ordinarily of the bulb type, and calibrated to deliver known amounts of fluid. Sometimes it is desirable to have a pipette with two calibrations, one to mark total *content* of fluid, the other to mark the

point to which the pipette must be filled to *deliver* the indicated volume, but usually only the delivery mark is needed. As in the case of burettes, accuracy requires that the outflow time and the diameter of the pipette stem at the calibration mark shall be related to the size of the pipette. Table 6 gives the minimum outflow times required by the Bureau of Standards (11) and the internal diameters of the stems at the calibration marks, for pipettes of different capacity.

The *outflow time* of the pipette can be controlled in two ways:

1. The bore of the tip may be so constricted that when the pipette is delivering freely the time required will be that given in table 6. Such pipettes are to be employed when the acme of accuracy is necessary; e.g., when standardizing volumetric solutions. All Bureau of Standards pipettes are for outflow in this manner.

TABLE 6

SPECIFICATIONS FOR PIPETTES

CAPACITY OF PIPETTE	MINIMUM OUTFLOW TIME	INNER DIAMETER OF STEM SUCH THAT ERROR OF 1 MM. IN LEVEL CAUSES ERROR OF 1 PART PER 1000 IN VOLUME MEASURED	RECOMMENDED INNER DIAMETER OF STEM AT CALIBRATION MARK
cc.	*seconds*	*mm.*	*mm.*
1	15	1.1	1
2	15	1.6	1
5	15	2.5	2
10	20	3.5	3
25	30	6.1	4
50	30	7.9	5
100	40	11.2	5

2. However, if many transfer have to be made, the time and effort required to draw the samples into the pipettes with such constricted tips become onerous. An alternative is offered in the use of *pipettes calibrated for retarded delivery*. These pipettes have outlets so large that they can be easily and quickly filled. If they are permitted to deliver freely they empty in ten seconds or less, with inaccurate drainage. However, accurate delivery is obtained if the outflow is retarded by keeping a finger over the upper opening, and permitting air to enter only at such a rate that the outflow of water requires the necessary fifteen to thirty seconds indicated in table 6. The outflow must also be so regulated that the descent of liquid in the pipette is steady. The finger used in closing the upper end of the pipette must be sufficiently moist to be soft, but must not be wet, in order properly to control the flow of liquid.

Calibration of pipettes for delivery. Pipettes are calibrated by weighing the water which they deliver into a weighing bottle which contains a layer of paraffin oil a few millimeters thick to prevent evaporation. If the mark is not accurate another mark is made with a wax pencil and tested. This procedure is repeated until the mark is correctly located. It is then etched in as described below.

If an uncalibrated pipette is to be marked, two preliminary marks separated by a definite distance, 50 mm. for example, are made on the stem, by strips of gummed labels or by a chisel-edged wax pencil, and the water delivered from each is weighed. From the difference, the weight of water contained in each mm. length of stem is calculated, and thence the number of millimeters from either preliminary mark to the correct mark. The latter is located at the calculated level on the stem, is tested by weighing the water delivered from it, and finally is etched in.

Example: The weights of water delivered at 20° by a 10 cc. pipette from two preliminary marks 50 mm. apart, are 9.900 and 10.275 grams. Hence the weight of the column of water between the marks is 0.375 gram or $\frac{0.375}{50} = 0.0075$ gram per millimeter of column length. From table 2 the desired delivery of water at 20° is 9.972 grams, or 0.072 gram more than that delivered from the lower preliminary mark. Hence the correct mark is $\frac{0.072}{0.0075} = 9.6$ mm. above the lower mark.

In calibrating a pipette, the same mode of regulating the outflow speed and terminating the delivery must be employed which is to be used later in transfer work.

Calibration of pipettes "to contain." The weight of water required to fill the dry pipette is measured. One may either weigh the pipette empty and full, or may fill it with water from a weighing bottle, which is weighed before and after. Other details are as for delivery pipettes.

Use of pipettes. The *delivery* of fluid from a pipette is made either *free*, or *retarded* by the finger tip, as described under "construction of pipettes," dependent on whether the tip is constricted or not.

For *terminating the delivery*, when all the fluid except the final drop has been delivered, three different methods are in use: viz., drainage, blow out, and delivery to a mark on the lower stem.

Drainage termination is the most convenient, and can be used for pipettes of 5 cc. or greater content without causing errors in excess of the 1:1000

limit. The tip of the pipette is allowed to touch the wet wall of the receiving vessel as delivery is finished. A drop of liquid remains held by capillary attraction in the tip of the pipette. The volume of this drop varies with the specific gravity, viscosity, and surface tension of the fluid, but with pipettes of 5 cc. or more capacity and the solutions ordinarily measured the variation is not significant. Drainage termination is used for all Bureau of Standard pipettes.

In *blow-out termination* the final drop is expelled from the tip of the pipette. The drop may be blown out with the breath when the solution measured provides no objection. The expulsion can also be conveniently effected by closing the upper end of the pipette with the right forefinger and warming the bulb by gripping it with the left palm. The expansion of air in the bulb forces the drop from the tip.

Delivery to a mark on the lower stem is an exact procedure, which will be outlined below in the description of special blood pipettes.

Each mode of delivery gives exact results under conditions suited to it. The essential point is that the same technique of delivery must be employed in the calibration of each pipette and in its subsequent use.

A rule which may be followed for ordinary pipettes is to employ drainage delivery for those above a certain capacity, and blow-out delivery or delivery to a mark for smaller ones. Unless some such rule, or uniform drainage or blow-out delivery for all pipettes, is used, each pipette must be etched "Drainage" or "Blow-out." In a laboratory where much use is made of pipettes too small (2 cc. or less) for precise drainage delivery, it is convenient to avoid confusion by calibrating *all pipettes for blow-out* delivery, except those delivering between 2 marks. Pipettes delivering between two marks are, of course, distinguished by the double calibration.

When corrosive or poisonous fluids are measured, a piece of cotton is placed in the upper stem of the pipette to prevent drawing the solution into the mouth.

MICROVOLUMETRIC APPARATUS AND ITS CALIBRATION

Microburettes. Burettes of from 3 to 0.1 cc. capacity can be made, in which the error of delivery does not exceed 0.001 of the capacity. For such burettes both the construction and the method of calibration must be different from those described above for standard burettes of greater capacity.

The internal diameters of such burettes must be smaller to permit the reading of finer subdivisions. The outlets must be of smaller bore and constructed to discharge smaller drops, or some other device must be used to facilitate delivery of finer subdivisions of fluids. In the smallest microburettes a substitute for stop-cocks is required to secure finer control of delivery.

Table 7 indicates the approximate volume of 1 mm. length of tubing of different diameters. This is the smallest distance between calibration marks compatible with easy reading and interpolation to 0.2 of an interval with the naked eye.

When the total volume delivered from a measuring apparatus is very small, accurate measurement demands that the fluid shall be delivered in minute drops, and that the amount adherent to the tip of the burette shall be insignificant. Both objects are attained to a considerable degree by using a delivery tip drawn out fine. Such tips, however, if of glass drawn very fine, are too flexible and fragile. By substituting small guage hypodermic needles, which are now manufactured from non-corrodible metals, Trevan and Bainbridge (15) have shown that drops of the order of 0.00015 cc. can be removed. This principle is utilized by Shohl (13) in the construction of his microburette tip, described below.

TABLE 7

VOLUME CONTENT PER MILLIMETER LENGTH OF TUBING OF DIFFERENT BORE

Internal diameter of tubing, mm.	10	9	8	7	6	5	4	3	2	1.5	1.0	0.9
Volume of 1 mm. length, cc.	0.08	0.06	0.05	0.404	0.03	0.02	0.013	0.007	0.003	0.002	0.0008	0.0006
Internal diameter of tubing, mm.	0.8	0.7		0.6	0.5							
Volume of 1 mm. length, cc.	0.0005	0.0004		0.0003	0.0002							

Another expedient to avoid the effects of variable adhesion to the tips of burettes in titrations is to keep the delivery tip immersed in the fluid which is being titrated. This is employed by Rehberg (12) in his microburette described below.

Stop-cocks provide only limited control of flow. By the introduction of thumb-screws working on inelastic reservoirs (see Rehberg pipette below) finer adjustment and control may be secured.

Bang's microburette.[2] The form of microburette in most common use is that introduced by Bang (1) and shown in figure 1. The burette is of 3 cc. capacity and about 4 mm. inner diameter. It is divided into 0.01 or 0.02 cc. divisions, and can be read to 0.002 cc. The bulb reservoir serves to hold

[2] The Bang microburette can be obtained from Machlett and Son, Glassblowers, 50 William Street, Long Island City.

The vertical cock shown in the illustration can be replaced by a horizontal cock, such as is ordinarily used in burettes. The horizontal cock could be provided with a Shohl tip (see below and fig. 2). The writers have not tested this addition to the Bang burette, but it appears that it would increase the instrument's sensitivity.

a convenient supply of solution, with which the burette tube is refilled after each titration. The sealed tube at the bottom of the burette tube may be fitted into a stand, so that the apparatus is a conveniently movable unit.

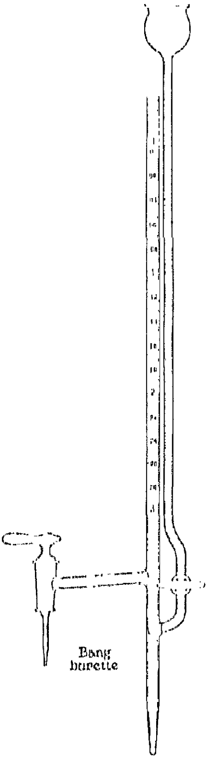


FIG. 1

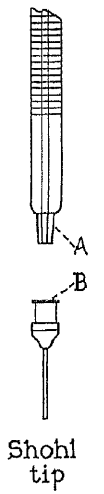


FIG. 2

FIG. 1. Bang microburette.

FIG. 2. Shohl needle tip for microburettes and pipettes. *A* shows a ground glass Luer adapter sealed to the outlet of a micro burette and *B* shows the Luer hypodermic needle with tip ground flat.

In the use of this burette it is essential to observe the rule that delivery should be so retarded that the surface of the liquid in the burette falls not faster than 0.5 cm. per second. Error due to incomplete drainage is rela-

tively greater in a microburette than in a large one, because the ratio of surface to volume is greater in the smaller tube.

For the same reason, errors in drainage due to grease on the burette wall are more important in the micro apparatus. It must be kept scrupulously clean.

The Bang burette can be calibrated by the same procedure described above for larger burettes: water in 0.2 cc. portions is delivered into a weighing bottle containing a layer of oil. There is no difficulty in making the weighings accurate to within less than 1 mg., which is an exact as one can read the level of the meniscus in the burette.

Calibration can also be made by weighing similar portions of mercury from the wet burette. For precautions necessary in calibrating with mercury, see section below on that subject.

The Shohl needle tip for microburettes and pipettes (13). (See figure 2.) This tip can be attached either to microburettes or to stop cock pipettes of the Van Slyke-Neill type described below. To the delivery end of such a burette or pipette a glass Luer adapter is sealed. The ground end *A* fits snugly into a Luer hypodermic needle B of 18 to 23 gauge which is cut off horizontally and ground on a stone. Needles of platinum or other noncorrosive metal are to be preferred. Tips of this type deliver extremely minute drops.

The Rehberg (12) microburette.[3] In this device, which is illustrated in figure 3, the fluid is delivered from a fine tip beneath the solution which is being titrated, under the control of a micrometer screw which activiates a column of mercury.

The burette itself consists of a capillary tube of 6 to 8 mm. outer diameter and a bore of 0.8 to 0.9 mm., so that the graduated portion, containing 0.1 cc. of liquid, is 150 to 200 mm. long. The tube is graduated into cubic millimeters by marks 1.5 to 2 mm. apart. Rehberg states that delivery can be measured to within 0.1 cu.mm., or 0.001 the total capacity of the burette. The tube is expanded at the lower end to receive a steel micrometer screw, which is sealed in with piccin or sealing wax. One revolution of the micrometer screw displaces about 4 cu.mm. The mercury is used to deliver aqueous solutions. The burette tip is composed of a separate glass capillary similar to that from which the burette is made. It is connected to the burette by means of a short, thick, rubber tube, supported by split glass tubing, which is held in place by a brass ring. The tip is made long enough to hold more solution than is used in a titration.

[3] The burette with stand can be purchased from the workshop of the Zoophysiological Laboratory, Ny Vestergade 11, Copenhagen, Denmark.

The movable arm, *6*, carries three test tubes: one filled with the fluid used for the titration; one a solution containing indicator showing the desired end-point; the third with the solution to be titrated.

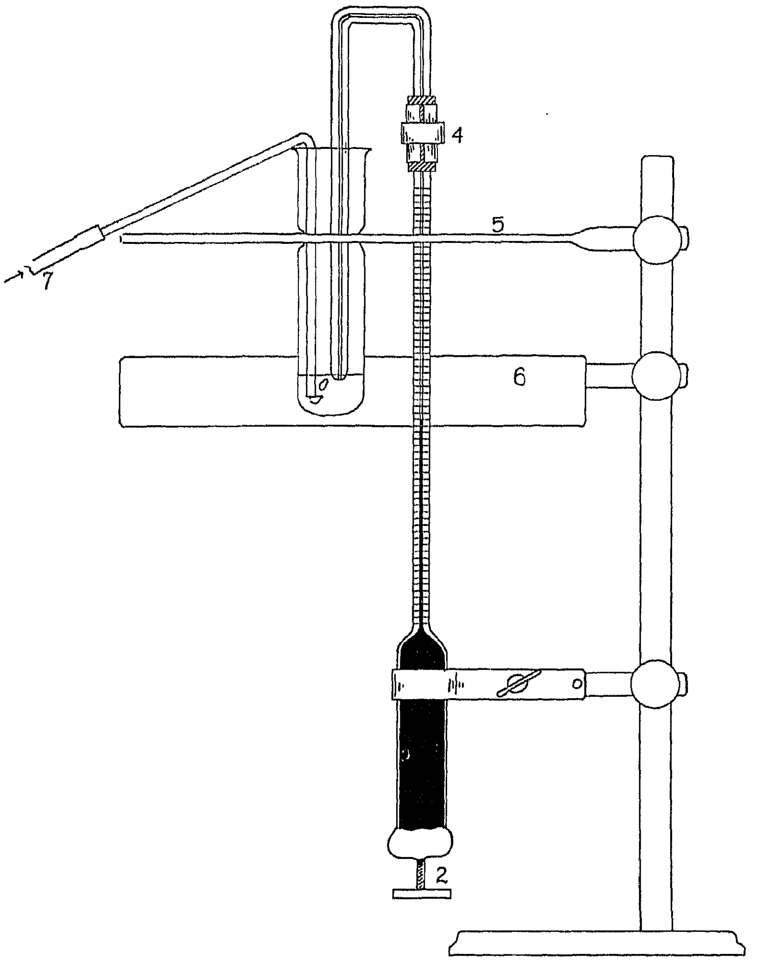


FIG. 3. *1*, Rehberg microburette; *2*, micrometer screw; *4*, connection between burette and detachable tip, made of rubber tubing reinforced by a split brass tube and held in place by a brass ring; *5*, tube holder for three tubes; *6*, white background: *7*, stirrer connected with source of air current.

To fill the apparatus for the first time, the tip is removed, the mercury is brought to the top of the burette, which is washed out once or twice and then filled with the titrating solution by means of a pipette and the micrometer screw. By a rubber tube attached to the separated tip, the latter is filled. It is then connected with the burette with care to avoid the introduction of air bubbles (a single bubble from 1 to 5 cu.mm. in volume which moves with the solutions causes no appreciable error). To refill the burette the tip is dipped into the fluid and the mercury is lowered.

The burette is filled until the mercury meniscus is slightly below the zero point, and the tip is wiped carefully with filter paper. Just before each titration the meniscus is brought to the zero point, and adherent fluid is removed from the tip with filter paper.

Because with such small volumes the indicator error is relatively large, the amount of indicator in all comparable titrations must be identical. During the titration the tip of the burette must dip just below the surface of the solution, which is stirred by a current of air bubbles.

If the burette is used for acidimetry the control tube is filled with a buffer solution which has the pH desired at the end point of the titration and which contains the same concentration of indicator as the unknown. In this case the air used for stirring must be freed from CO_2 by soda lime. It is convenient to use as a source of air a 2- to 5-liter aspirator bottle containing sodium hydroxide solution, which effectively removes the CO_2.

Rehberg calibrated his burette by measuring the length of a weighed amount of mercury at different positions in the tube. This is, however, a tedious procedure involving calculations not at all simple,[4] and it makes no allowance for the volume of water film left adherent to the walls of the tube when the burette is used for delivery of aqueous solutions. A simpler procedure is to deliver from the burette 2 N sulfuric acid in portions of 10 cu.mm. (0.01 cc.), and titrate them in the Rehberg cup with freshly prepared 0.1 N alkali from a calibrated Bang microburette. The cubic centimeters of 0.1 N alkali multiplied by 50 indicate the cubic millimeters of 2 N acid delivered by the pipette. The correction at any point on the Rehberg burette is calculated as: $50A-$(cubic millimeters on scale of burette) = correction in cubic millimeters. A = cubic centimeters of 0.1 N alkali used.

Presumably the Rehberg burette, without the delivery capillary, could be inverted and calibrated by means of a micro-modification of the device shown in figure 5 and described below under "Calibration with mercury."

[4] Hulett (7) has described a simpler procedure, in which successive equal portions of mercury are withdrawn from the graduated capillary by means of a still finer capillary, marked for definite content. This procedure requires some technical skill.

Blood pipettes. For micro blood analyses portions of 1 or 2 cc. are commonly measured in transfer pipettes. With such small pipettes the fact, that the surface is relatively large compared with the volume, increases the possibilities of error due to variations in the thickness of fluid film left adherent to the walls, and in the amount of fluid adherent inside and outside the pipette tip at the end of delivery.

To minimize the amount and variability of the film left adherent to the walls, it is necessary to limit the rate at which the pipettes empty. It is impractical to retard the flow adequately by constricting the bore of the tip, because tips of the necessary fineness are likely to clog when used with blood. The retardation is preferably accomplished by the technique of "retarded delivery" previously described for pipettes in general. The tip is made of sufficiently wide bore to permit easy filling and freedom from clogging, and the rate of delivery is controlled either by a finger over the upper stem, or by a stop-cock constructed in the lower stem (see fig. 30, p. 240). The rate of delivery can thus be kept slow enough to assure good drainage of the film from the walls. In measuring whole blood it must not, however, be so slow that cells and serum separate appreciably during the delivery: such a separation would cause the discharged fluid to have a slightly greater proportion of cells than the mixed blood, the fluid retained in the pipette having a greater proportion of serum.

To avoid variation in the quantity of fluid retained in the tip, one may use blow-out delivery.

The authors have obtained, however, the most satisfactory results in blood analyses by the use of specially constructed pipettes of the Ostwald type, calibrated to *deliver between two marks*. With these blood can be delivered quantitatively with an error not exceeding 0.1 per cent from pipettes of 2 to 5 cc. capacity, and with only 0.2 per cent error from 1 cc. pipettes. For smaller quantities the percentage error is proportionally greater. Such a pipette can be discharged with the tip under fluid, a procedure which eliminates variations in the quantity of fluid adherent to the tip of the emptied instrument, and furthermore permits the measurement and delivery of blood anaerobically for gas analysis.

The general construction of such pipettes is illustrated in figure 30, in the chapter on gasometric methods, p. 240. For many purposes the stop-cock is not required, but it facilitates accurate delivery.

The upper and lower stems of the pipettes are made of heavy walled tubing with an inside diameter of about 1 mm., in which 0.001 cc. of fluid makes a column 1.2 mm. high. The upper mark should be from 1 to 6 cm. above the upper end of the bulb, the lower mark at least 1 cm. and not more than

3 cm. below the lower end of the bulb. If there is no stop-cock the delivery tube below the mark should be 8 cm. long. If there is a stop-cock this should be attached about 2 cm. below the lower mark and the delivery tube below the cock should be 8 cm. long. The lower end is tapered and the tip ground smooth. The orifice in the tip should be large enough to permit blood to flow, but small enough so that, when the flow is unretarded, the water meniscus will not fall more rapidly than 0.5 cm. per second in the bulb (it will of course fall much faster in the capillary stems). Stop-cocks, when used, should be of 1 mm. bore. The bulb is constructed of thick glass of such a form that there are no shoulders to retain fluid and no constrictions or irregularities at the junctions with upper and lower stems.

In calibrating such pipettes water is drawn to the mark in the usual manner. It is then discharged into a weighing bottle containing paraffin oil at such speed (controlled by the finger or the stop-cock) that the meniscus falls at no time more rapidly than 0.5 cm. per second. When the meniscus is still about 1 cm. above the lower mark, the tip is brought into contact with the oil in the weighing bottle and kept there until the water level reaches the lower mark, when delivery is stopped.

Pipettes of small capacity, 1 cc. or less, can also be rapidly and accurately calibrated with water over mercury with the aid of the bulb and stop-cock described below under "Calibration with mercury" and illustrated in figure 5.

For some purposes pipettes are made and calibrated *to contain* rather than to deliver a given quantity of fluid. The use of a pipette calibrated "to contain" is permissible, however, only if the residual fluid, left in the pipette after drainage is complete, can be rinsed into the part first delivered.

CALIBRATION OF VOLUMETRIC APPARATUS WITH MERCURY

Since mercury at room temperature weighs 13.57 times as much as the same volume of water, small volumes of the metal can be weighed with greater percentage accuracy. Also there is no danger of loss by evaporation during weighing, nor need precautions be taken with regard to drainage, or to adhesion of the metallic fluid to tips of burettes. For these reasons mercury is frequently preferable to water for calibration of micro-apparatus.

In changing from water to mercury, however, one must exercise not fewer, but different, precautions to avoid inaccuracies. As a rule, apparatus of 0.5 cc. capacity or greater can be more satisfactorily calibrated as previously described with water.

Three peculiarities of mercury must be taken into consideration: 1, its convex meniscus; 2, its failure to wet glass; 3, the greater pressure exerted by a long column.

1. If a vessel is intended for the measurement of aqueous fluid, with a concave meniscus, the use of uncorrected calibration readings obtained with the convex meniscus of mercury will introduce an error, unless a meniscus of equal diameter is observed both before and after delivery. Such menisci are observed in burettes, and in pipettes which deliver between two marks when both marks are on stems of like bore. These can accordingly be calibrated with mercury, and the uncorrected marks used to measure water. But in dealing with other apparatus (e.g. pipettes calibrated "to contain" definite volumes when filled from the tip, and Van Slyke-Neill extraction chambers, p. 270), errors from the opposite curvatures of the 2 menisci may be significant.

Since the mercury meniscus curves downwards from the center, a pipette filled with mercury, so that the center of the meniscus is on a given mark, will contain less mercury than is indicated by the volume content of the pipette up to the level of the mark. On the other hand, since the water meniscus curves *upwards* away from the mark, when the pipette is filled with water so that the center of the meniscus is on the same mark, the volume of fluid present will be greater than the pipette would contain if filled just level with the mark.

Consequently when a vessel is calibrated with mercury to contain water, a correction must be made for the sum of the two so-called meniscus errors in opposite directions. One must weigh into such a vessel a volume of mercury *less* than the volume of water for which the vessel is to be used. The wider the diameter of the tube where the mark is placed, and the greater the meniscus, the larger will be the absolute correction. The magnitude of the correction for tubes of different diameters is given in figure 4.

For example, if a straight micropipette of 1.5 mm. bore is calibrated with mercury to contain 0.2 cc. of blood or water, the curve of figure 3 indicates that 0.0014 cc. less than 0.2 cc. of mercury must be weighed into the pipette. Therefore instead of $0.2 \times 13.55 = 2.710$ grams of mercury one weighs $0.1986 \times 13.55 = 2.690$ grams.

With a meniscus of given diameter, the significance of the correction varies inversely as the volume of solution measured. The above correction for a 1.5 mm. meniscus is significant for a 0.2 cc. pipette, but for a 2.00 cc. bulb pipette with a stem also of 1.5 mm. bore the correction of 0.0014 cc. would not ordinarily be significant.

2. Mercury does not wet glass and therefore leaves no film behind it, as water does. Consequently a dry burette delivers a greater volume of

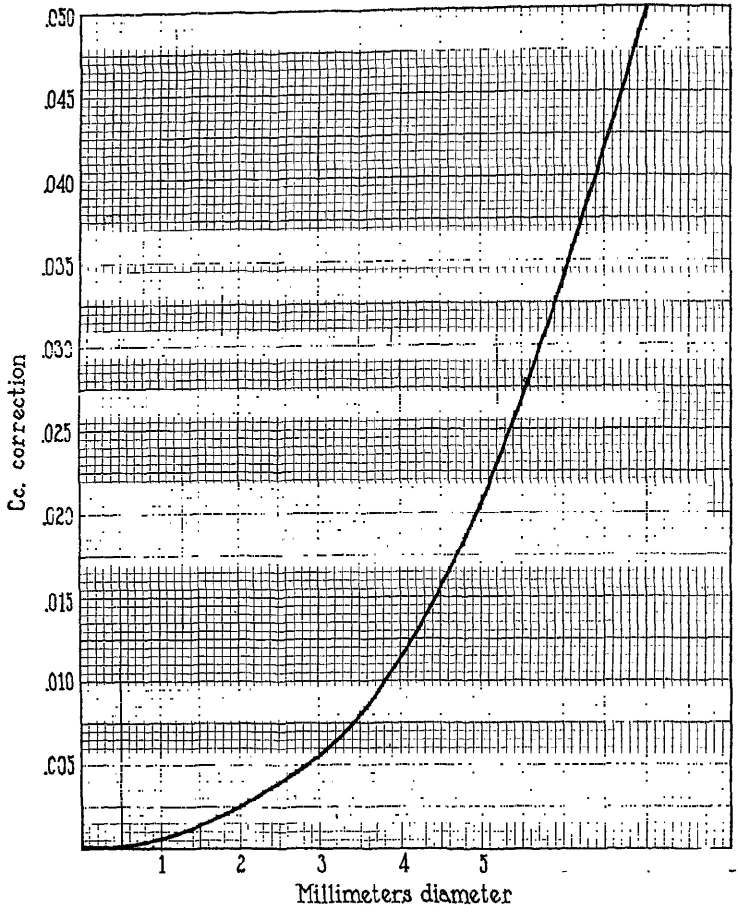


FIG. 4. Correction for difference between water and dry mercury menisci in tubes of different bore. The data for tubes of more than 4 mm. bore are taken from table IV of Lunge-Berl's "Chemisch-technische Untersuchungsmethoden," vol. 1, p. 75, Berlin, 1921. The data for tubes of less than 4 mm. diameter were obtained by the writers, by comparison of calibrations with water and mercury.

mercury than of water, of which a small part remains as the adherent film. Apparatus which is for *delivery* of water solutions should be wet with water before it is filled with mercury for calibration.

3. The weight of mercury is so great compared with its cohesion that delivery tips must be made extremely small or the mercury near the orifice will tend to drop out. Mercury drops from such a tip should be about 0.005 cc., or 0.07 gram. In microapparatus, delivery tips are usually of such small bore that no special provision need be made against this source of error. With apparatus of coarser bore, a separate stop-cock with a fine delivery tip must be attached. If this connection is made by means of rubber tubing, even wired pressure tubing, the weight of mercury is so

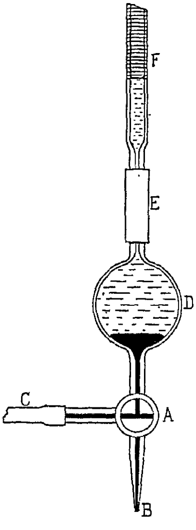


FIG. 5. Bulb and three-way stop-cock for use in calibration of burettes and pipettes with water over mercury. *A*, 3-way stop-cock; B, fine delivery tip; *C*, connection with mercury leveling bulb; *D*, bulb of thick glass with capacity larger than that of burette; *E*, connection of heavy pressure tubing.

great that the tubing will expand steadily as the column rises, and contract again as it falls. To obviate error from this source a special calibrating cock may be sealed to such apparatus, or a device like that illustrated in figure 5 may be used.

Burettes should not be filled for calibration by introducing mercury from above, because in falling it is likely to trap air bubbles. The mercury should be driven in from below by pressure from a leveling bulb attached to the outlet by rubber tubing, or should be drawn into the burette or pipette by suction.

The usual type of microburette with a fine enough tip to sustain mercury can be calibrated very simply. Clean mercury is placed in a beaker. The delivery tip of the burette is immersed in the mercury; the other end is attached to a water pump by means of pressure tubing equipped with a screw clamp or, better still, a three-way stop-cock by which the vacuum can be turned on or off at will. If the burette is to be used to deliver aqueous solutions, a small amount of water is drawn into the tip of the burette before this is placed in the mercury. Suction is applied and the mercury is drawn slowly up into the burette until the meniscus is above the zero mark. The burette stop-cock is now closed, the vacuum is cut off and, by means of a fine drawn out capillary tube, or a roll of filter paper, the water floating on top of the mercury is removed. The mercury meniscus is then brought down to the zero point. The beaker of mercury is removed from below, and a tared weighing bottle is put in its place. Fractional volumes of mercury are weighed in the manner described above for the calibration of standard burettes with water.

(Grams Hg) $\times$ (Cubic centimeter volume of 1 gram Hg) = volume in cubic centimeters

The volume in cubic centimeters of 1 gram of mercury is taken from the last column of table 2.

If the burette or apparatus is not equipped with a stop-cock at the bottom or with a fine enough tip to support a column of mercury, and it is desired to calibrate it with water over mercury, the apparatus illustrated in figure 5 is convenient. The three-way stop-cock *A* has as one arm a fine capillary tip which will sustain mercury; the other arms are connected to a mercury leveling bulb *C* and a mercury reservoir *D*. The reservoir must have a capacity slightly greater than that of the burette it is intended to calibrate and must be made with heavy enough walls to withstand filling with mercury.

To the upper end of the bulb is attached by heavy walled tubing ***E*** the burette to be calibrated. The two glass ends within the tubing should be as close together as possible. Water is drawn through ***B*** until the bulb is almost completely filled. With the leveling bulb elevated, mercury is now introduced until the water has been displaced into the burette to a point somewhat above the zero. Mercury is wasted through ***B*** to drive out the residual water, and the tip is carefully blotted to remove adherent droplets. Calibration is now carried out in the usual manner. Mercury is delivered through ***B*** and weighed, while measurements are made with the water meniscus in the burette. The rubber tubing is called upon to resist only the pressure of a water column. The whole apparatus must be rigidly set up so that move-

ments of the stop-cock and leveling bulb do not cause flexion or stretching of the rubber joint *E*.

For *calibrating a capillary tube* of over 0.5 mm. bore, by measurement in a still finer capillary of successive portions of mercury withdrawn from the tube, the method of Hulett (7) has already been mentioned. It is not required for any of the apparatus used for analyses described in this volume. Hence the reader who may have occasion to use it is referred to the original article (7).

STANDARD VOLUMETRIC SOLUTIONS

Definition of molar and normal solutions for volumetric analysis

A molar solution of any chemical compound, in the terminology of analytical chemistry, is a solution of such a concentration that 1 liter contains one gram molecule of the compound, e.g., the molecular weight of hydrochloric acid, HCl, is 36.46. Therefore, a molar solution of hydrochloric acid contains 36.46 grams of HCl per liter; a molar solution of sulfuric acid, H_2SO_4 (molecular weight 98.08) contains 98.08 grams per liter.

A *normal solution* of any compound is a solution which contains 1 gram atom (1.008 grams) of reacting hydrogen per liter, or which can quantitatively replace or react with an equal volume of such a solution. *The fraction of a gram molecule of any substance in a liter of normal solution depends upon the reaction for which the substance is to be used.*

In *acid-alkali titration* a normal acid solution contains per liter the amount of acid that has 1 gram atom of hydrogen replaceable by alkali at the pH used as end point in titration; e.g., one molecule of hydrochloric acid, HCl, contains one atom of replaceable hydrogen, therefore a liter of a normal solution of this acid contains 1 gram molecule, or 36.46 grams of HCl. One molecule of sulfuric acid, H_2SO_4, contains, however, for titration to ordinary end points, 2 atoms of replaceable hydrogen; therefore a liter of normal sulfuric acid contains one-half of a gram molecule, or $0.5 \times 98.08 = 49.04$ grams of H_2SO_4. In the case of phosphoric acid, 1 hydrogen is neutralized by titration to pH 5, 2 by titration to pH 9, and 3 by titration to pH 12. A normal solution of phosphoric acid would, therefore, contain one mole (mole = gram molecule) per liter if it were to be used for titration to pH 5.0, $\frac{1}{2}$ mole per liter if the end point were pH 9, and $\frac{1}{3}$ if it were to be 12. In practice such an acid is not used for a standard. Its behavior serves to illustrate, however, that in acidimetry one must use as end point a pH suited to the strength of the acid, strong acids being accurately titratable at pH as low as 5, while in weak acids (most organic ones), the acid H is completely re-

placed by alkali only at higher pH. Hence the alkaline end point (pH 8.0–8.5) of phenolphthalein is customarily used for organic acids.

A normal *alkali* solution is one which will neutralize, volume for volume, a normal acid solution. A normal solution of NaOH is molar, but one of $Ba(OH)_2$ is half molar.

A normal *reducing* solution is one of which a liter contains 1 gram atom of oxidizable hydrogen or its equivalent in other reducing substances. Oxalic acid, $H_2C_2O_4$, has 2 hydrogen atoms, both of which are titratable with alkali, and both of which are oxidizable by permanganate. Hence a normal solution of oxalic acid, whether for acidimetry or for oxidation by permanganate, is half molar.

A normal *oxidizing* solution is one of which a liter will oxidize 1 gram atom of hydrogen, or its equivalent of other reducing substances. In the reaction

$$2\ KMnO_4 + 5\ H_2C_2O_4 + 3\ H_2SO_4 = K_2SO_4 + 2\ MnSO_4 + 10\ CO_2 + 8\ H_2O$$

each molecule of permanganate oxidizes 5 of the hydrogen atoms of oxalic acid. Hence a normal solution of permanganate is only fifth molar.

General principles governing the preparation and maintenance of standard solutions for volumetric analyses

Certain standard solutions can be directly and accurately prepared by weight. Others are standardized directly or indirectly against those which have been prepared by weight. All solutions are so prepared that a certain quantity of solute is contained in a known volume. The usage in physical chemistry, of considering concentrations in terms of moles of solute per mole of solvent, rather than per volume of solution, is impracticable for solutions used in volumetric analysis.

In general, volumetric standards, like volumetric measuring apparatus, should be accurate to 1 part in 1000. It may be convenient, especially if the standard is subject to deterioration or variation in strength, instead of adjusting it to the exact strength desired, to bring it approximately to that strength, and to use in calculations a factor which relates the actual strength of the solution to the strength of the theoretical standard. For example, if 50 cc. of accurate 0.1 N standard hydrochloric acid, made from Hulett and Bonner acid (see below), is neutralized by 49.5 cc. of alkali, each cc. of alkali has an actual strength of

$$\frac{50}{49.5} \text{ of } 0.1\ \text{N; or the factor, } f = \frac{50}{49.5} = 1.010, \text{ for the NaOH solution}$$

Multiplying the volume of alkali used in a given titration by this factor, f, gives the volume of exactly 0.1 N alkali that would be used.

TABLE 8

STRENGTHS OF CONCENTRATED COMMERCIAL SOLUTIONS OF ACIDS AND ALKALIES

ACID OR ALKALI	TRADE SYMBOL OF STRENGTH	SPECIFIC GRAVITY	GRAMS ANHYDROUS SUBSTANCE PER 100 GM. SOLUTION	GRAMS PER LITER	GRAM MOLECULES PER LITER	CUBIC CENTIMETERS REQUIRED TO MAKE 1 LITER OF 1 N SOLUTION
Hydrochloric acid (HCl)	Concentrated	1.19	36–37	440	12	83
Sulfuric acid (H_2SO_4)	Concentrated	1.84	94	1730	18	28
Nitric acid (HNO_3)	Concentrated	1.42	69–70	990	16	64
Phosphoric (ortho) acid (H_3PO_4)	Syrupy	1.71–2	85	1450	15	67 or 34†
Lactic acid ($CH_3 \cdot CHOH \cdot COOH$)	Concentrated	1.21	85	1030	11	87
Acetic acid ($CH_3 \cdot COOH$)	Glacial	1.06	99.5	1060	17–18	57
Sodium hydroxide ($NaOH$)	Concentrated	1.50–53	41–47	600 to 700	15–17	57–67
Potassium hydroxide (KOH)	Concentrated*	1.55	51	800	14	70
Strong ammonia water (NH_4OH)	Concentrated	0.90	28 (NH_3)	250	15	67

* Saturated solutions made from the usual C.P. potassium hydroxide will vary in strength, chiefly because of the variable amount of carbonate which such preparations contain.

† 67 cc. of concentrated phosphoric acid per liter are used to make a normal solution when only 1 acid hydrogen atom is to be titrated, with an end point of about pH 5. When 2 acid hydrogen atoms are to be titrated, with an end point of about pH 8.2, half as much phosphoric acid per liter is required to make a normal solution.

Table 8 gives the strengths of concentrated acid and alkali solutions commonly used in commerce, and the quantities of each solution which, if diluted to 1 liter, will yield an approximately 1 N solution.

Preservation of standard solutions

Solutions vary in durability and stability. Some must be restandardized daily to correct *f*, some every three months; and some remain unchanged indefinitely. It is well to mark on the labels of all containers of standards the dates of preparation and restandardization, with the value of *f* obtained on each occasion.

Some standard solutions tend to become contaminated with molds and other impurities, even when they do not deteriorate as a result of contamination with carbon dioxide, oxidation, exposure to light, changes of temperature, or solution of alkali and silicates from glass. Certain precautions can be employed to minimize these sources of contamination and deterioration.

Only the highest quality chemicals, which have been kept in closed containers free from dust, are used; and these are exposed to the air no longer than is absolutely necessary for the preparation of the reagent desired. No instrument or foreign matter should be introduced into a reagent container unless necessary, and then only if such instrument is scrupulously cleaned and composed of material that will not react with the reagent. Because of the possibility that they may have become contaminated, reagents or chemicals that have been removed from stock containers for any purpose should not be returned to these containers. It follows that one should remove from stock containers no more material than is necessary for immediate requirements. If an excess is removed and can not be wasted, it should be placed in a new clean container. Necks and stoppers of containers should be carefully cleaned of dust before the containers are opened.

It is a good general precaution to keep stock standard solutions in a refrigerator. Cold retards the chemical reactions which may deteriorate the solutions. It also retards growth of molds and bacteria.

Photosensitive solutions should be kept in dark glass.

Addition of disinfectants and preservatives is to be avoided if possible, but may be necessary for some solutions.

Preparation of standard hydrochloric acid solution from the constant boiling mixture method of Hulett and Bonner (3, 5, 6)

This method, which is capable of accuracy to 1 part in 10,000, depends on the fact that when hydrochloric acid solution is distilled the concentration

of acid in the undistilled portion approaches a constant concentration (20.22 per cent of HCl by weight after distillation at 760 mm. pressure). If boiling is continued water and hydrochloric acid distil off in constant proportions identical with those in the residual undistilled fraction.

To concentrated hydrochloric acid (sp. gr. 1.2) add an equal volume of water. Bring the solution to a density of 1.096 at 25° by the addition of more water or acid. Distil away three-quarters of the mixture at the rate of 3 or 4 cc. per minute. The remaining one-quarter has, within 1 part in 10,000, the composition given in table 9. Of this quarter all but the last 50 or 60 cc. is distilled, and the distillate used to prepare

TABLE 9

HULETT AND BONNER'S CONSTANT BOILING HYDROCHLORIC ACID (6)

Values for distillation pressures 730–770 corrected by Foulk and Hollingsworth (5). Values for distillation pressures 620–660 added by Bonner and Branting (3).

BAROMETRIC PRESSURE AT TIME OF DISTILLATION	HCl CONCENTRATION BY WEIGHT	SOLUTION REQUIRED TO MAKE 1 LITER OF 0.1 N HCl
mm. Hg	*per cent*	*grams*
620	20.560	17.719
630	20.532	17.743
640	20.504	17.767
650	20.471	17.800
660	20.438	17.824
730	20.293	17.956
740	20.269	17.977
750	20.245	17.998
760	20.221	18.019
770	20.197	18.041

standard solutions. The barometer is recorded at the time of the distillation. In glass-stoppered bottles such stock solutions keep indefinitely.

The dilute standards are prepared by diluting the proper weight (not volume) of the acid, estimated from table 9, to volume with water. 16.4 cc., approximating 18 grams, of the acid are measured into a 50-cc. flask. More acid is added or withdrawn, by means of a medicine dropper with a fine drawn out tip, until the exact weight in table 9 is obtained. The acid is then diluted with water, rinsed into a 1-liter calibrated flask, and diluted to volume. Further standardization is unnecessary. In fact, acid prepared in this manner can be relied upon for the standardization of alkali and other reagents.

Standardization of acid gravimetrically with calcite (Iceland spar)

Acids which form soluble calcium salts can be standardized gravimetrically with accuracy against calcite, an extremely pure crystalline form of calcium carbonate.

To a weighed crystal of Iceland spar in a beaker is added a known volume, 50 to 100 cc., of the acid to be standardized. The beaker is is covered with a watch glass and set aside until the action of the acid on the crystal has completely ceased. The latter is removed from the solution, washed in distilled water, dried, and reweighed. The loss of weight is due to the solution of the carbonate under the influence of the acid.

$$\frac{200 \times \text{(grams of Iceland spar dissolved)}}{\text{cubic centimeters of acid added}} = f \text{ for } 0.1 \text{ N acid.}$$

If the acid is made up somewhat stronger than 0.1 N, and a given volume of it is diluted to f times that volume, the diluted acid will have a factor of exactly 1.

Standardization of other acids by comparison with standard hydrochloric acid

Hydrochloric acid standards prepared by weight from Hulett and Bonner acid, as described above, may be used to standardize any other acid solution by the following procedure.

With a pipette measure 20 or 25 cc. of standard hydrochloric acid into a flask and titrate from a burette with alkali of approximately the same normality. With the same pipette the same volume of the other acid is measured into a flask and titrated, using the same quantity of the same indicator, with the same alkali from the same burette, which is filled exactly to the zero point at the beginning of each titration.

The factor of the unknown acid is calculated as

$$f = \frac{\text{cubic centimeters standard HCl}}{\text{cubic centimeters of other acid}}.$$

This procedure eliminates errors from calibration of different pieces of glassware and from deterioration or inaccurate standardization of the alkali.

Preparation of concentrated sodium hydroxide approximately free from carbonate

Dissolve a given weight (100 to 500 grams) of "chemically pure" sodium hydroxide in an equal weight of water. The solution becomes very hot, and to avoid breakage should be made only in a vessel of Pyrex glass, or a procelain or earthenware jar. After the solution has cooled it is transferred to a paraffin lined bottle, which is kept tightly stoppered with a paraffined cork. Sodium carbonate is almost insoluble in such strong hydroxide, and therefore whatever carbonate is present, except traces that are negligible for most purposes, settles in a few days to the bottom as sediment. Portions of the clear supernatant fluid are withdrawn and diluted to make standard alkali solutions.

The concentrated solution contains about 70 grams of NaOH per 100 cc. It is used in the preparation of 0.1 N alkali described below.

Preparation of 0.1 N sodium hydroxide

For each liter of 0.1 N sodium hydroxide desired, dilute 5.9 cc. of the clear supernatant concentrated alkali, measured with a graduated pipette, to a liter. If a standard solution with the extreme minimum of carbonate is required, the dilution is performed with water which has been freed of CO_2 by acidifying with sulfuric acid and distilling to obtain a distillate free of ammonium carbonate. This distillate is freed of remaining traces dissolved CO_2 by boiling or by passing through a current of air which has been freed of CO_2 by passage through efficient soda lime towers. For ordinary alkali-acid titration work, however, the usual distilled water is sufficiently pure.

To standardize the 0.1 N alkali solution, 20 or 25 cc. of 0.1 N standardized acid are measured with a calibrated pipette into a flask, indicator is added, and the alkali solution is run in from a burette until the end point is reached. In titrating alkali against acid it is usually preferable to run the alkali into the acid, rather than the reverse, for two reasons: 1, the alkali has less opportunity to absorb CO_2 from the air than it would if it were first measured into the flask and kept there, with shaking, during the titration; 2, when alkali is added to acid the commonly used indicators change from colorless to colored, or from a lighter to a more intense color, changes that are more easily detected than the reverse.

When the titration in duplicate has been completed, the 0.1 N factor of the alkali is calculated as

$$f = \frac{\text{cubic centimeters 0.1 N acid}}{\text{cubic centimeters alkali}}.$$

All permanganate solutions should be restandardized at intervals, as they tend to decompose slowly, especially if they are exposed to light. If protected from light by being kept in dark bottles in a cupboard, however, 0.1 N solutions may maintain their strength for months.

Permanganate solutions more dilute than 0.1 N are made up from the 0.1 N by dilution in small quantities. They should be standardized on the day they are to be used, by titration against standard oxalic acid or sodium oxalate solution of similar strength, prepared by dilution of 0.1 N solutions.

Preparation of 0.1 N iodate or biiodate solution for the standardization of thiosulfate

Potassium iodate, KIO_3, or potassium biiodate, $KH(IO_3)_2$, can be secured in extremely pure form (Merck's "reagent") and used for the preparation of stable solutions for the standardization of thiosulfate.

Each molecule of iodic acid or iodate, when reacting with excess potassium iodide in the presence of acid, liberates 6 atoms of iodine by the reaction,

$$2\ KIO_3 + 10\ KI + 12\ HCl = 12\ KCl + 6\ H_2O + 6\ I_2.$$

Therefore, a liter of normal solution of iodate or biiodate contains one-sixth of a gram molecule. Of potassium iodate, KIO_3, with a molecular weight of 214.0, a liter of 0.1 N solution contains $\frac{21.40}{6} = 3.567$ grams. Of potassium biiodate, $KH(IO_3)_2$, with a molecular weight of 389.97, a liter of 0.1 N solution contains $\frac{38.997}{12} = 3.250$ grams.

Weigh 3.567 grams of potassium iodate or 3.250 grams of potassium biiodate. Dissolve the salt in a small amount of water, transfer the solution quantitatively to a liter volumetric flask and dilute to the mark with water. The solution, in a glass-stoppered bottle, in a cool place, will retain its strength almost indefinitely.

Standardization of 0.1 N thiosulfate solution

In the titration of sodium thiosulfate against iodine the reaction is

$$2\ Na_2S_2O_3 + I_2 = 2\ NaI + Na_2S_4O_6$$

One molecule of thiosulfate is oxidized by one atom of iodine. Therefore, a one-tenth molecular solution of thiosulfate is tenth-normal with respect to its reaction with iodine. The molecular weight of crystalline sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$, is 248.2. Therefore 24.82 grams of the salt are diluted to a liter to make a 0.1 N solution.

The purity and crystal water content of sodium thiosulfate are not sufficiently constant to ensure the accuracy of a standard solution thus prepared by weight. It must be standardized by titration against standard iodate or permanganate solution. The thiosulfate solution may keep unchanged for several months, but it is not entirely stable, and should consequently be restrandardized at intervals.

It may be standardized by titration against standard solutions of iodate, iodine, or permanganate. The iodate is the simplest and most satisfactory.

As an alternative we describe the permanganate method also. Because of the inconvenience of preparing standard iodine solutions by weight, and their instability, we do not include its use for standardizing thiosulfate.

Standardization of thiosulfate by titration against iodate or biioate

To 25 cc. of the standard 0.1 N iodate or biiodate solution in an Erlenmeyer flask add about 10 cc. of a 10 per cent solution of potassium iodide and about 20 cc. of 1 N sulfuric or hydrochloric acid. The thiosulfate is delivered into the acid iodate from a burette. When the iodine color has faded to a pale yellow 1 cc. of starch solution, prepared as described below, is added and the titration is continued until the blue color of the starch-iodine compound has entirely disappeared.

$$\frac{\text{cubic centimeters of 0.1 } N \text{ iodate}}{\text{cubic centimeters of thiosulfate}} = f \text{ for 0.1 } N \text{ thiosulfate.}$$

Standardization of thiosulfate against permanganate

One or two grams of pure potassium iodide, dissolved in a minimum of water in an Erlenmeyer flask, are acidified with 5 cc. of dilute hydrochloric acid (1 volume of concentrated hydrochloric acid diluted with 5 volumes of water). Twenty to 25 cc. of 0.1 N potassium permanganate solution are then added, which liberate an equivalent amount of free iodine from the iodide. The solution is at once titrated with thiosulfate, with starch indicator used in the manner described above.

$$\frac{\text{cubic centimeters of 0.1 } N \text{ permanganate}}{\text{cubic centimeters of thiosulfate}} = f \text{ for 0.1 } N \text{ thiosulfate.}$$

Thiosulfate solutions more dilute than 0.1 N are prepared by dilution from the 0.1 N solution and should be standardized on the day they are used, by titration against an iodate or biiodate solution of equivalent strength prepared by dilution of 0.1 N iodate or biiodate.

Preparation of 0.1 N iodine solution

For each liter of solution desired 13 grams of iodine, instead of the theoretical 12.69 grams are weighed into a weighing bottle. About 30 grams of pure potassium iodide are dissolved in about 250 cc. of water. The iodine is transferred from the weighing bottle to a 1 liter volumetric flask, any crystals of iodine which cling to the walls of the weighing bottle being washed into the flask with the iodide solution. The remainder of the iodide solution is then poured into the flask, the contents are shaken until all the iodine has dissolved, and the solution is diluted to volume with water.

The iodine solution is standardized by titrating 20 cc. with thiosulfate with starch indicator, in the manner described above for standardization of thiosulfate against iodate.

$$\frac{\text{cubic centimeters of 0.1 } N \text{ thiosulfate}}{\text{cubic centimeters of iodine solution}} = f \text{ for 0.1 } N \text{ iodine.}$$

Starch solution for iodometric titrations

A 1 per cent solution may be made by dissolving 1 gram of a high grade soluble starch (Merck's Lintner soluble starch is excellent) in cold water and diluting to 100 cc.

A solution may also be prepared from ordinary corn or potato starch. The latter has been found uniformly satisfactory. One gram of starch is rubbed up with a little cold water until a suspension has been formed. This is poured into 200 cc. of boiling water and boiled for a few minutes. The mixture is allowed to stand until the sediment has settled. The clear supernatant solution is used as the indicator.

Starch solution may become mouldy and deteriorate after a few days. The deterioration is prevented or greatly retarded by adding 1 gram of salicylic acid per liter to the water used for preparing the starch solution.[5]

ACCURACY IN VOLUMETRIC ANALYSIS

Even when each piece of apparatus and each standard solution used is accurate to 1 part per 1000, such precision can seldom be claimed for a volumetric analysis. A burette which delivers 50 cc. with an accuracy of 1 part per 1000 does not deliver 10 cc. with such precision. The allowed error of

[5] C. L. Alsberg and E. P. Griffing (*J. Am. Chem. Soc.*, 1931, 53, 1401) state that clear starch solutions for iodometric titrations can be conveniently prepared merely by extracting with water the well known breakfast food, puffed rice.

0.05 cc. constitutes not 1, but 5 parts per 1000 when only 10 cc. are measured. Furthermore the total possible error of a titrimetric analysis is the *sum* of the errors in each piece of apparatus, plus that in each standard solution, plus that involved in judging the end point of the titration. It follows that, other things being equal, that method of analysis will yield the most accurate results which requires measurement of the fewest liquid volumes, use of the fewest standard solutions, and determination of only one titration end point.

Usually the *errors in measurements of solution volumes and in fixing the end point* are constant absolute amounts, not constant percentages. There is a certain fraction of a cubic centimeter error in the delivery of each pipette or burette, and in the fixation of the end point. The added error of these factors in the final result of the analysis is therefore represented by ±milligrams or millimoles of the substance determined, rather than by a percentage of it. *The percentage error from measurements is least when the sample analyzed contains the largest amount of substance that can be titrated with one filling of the burette used.*

The *errors due to standardization of the standard solutions used,* on the other hand, are constant percentages of the amounts used and can not be decreased by taking larger samples of material for analysis.

As an example of calculation of possible error in a common volumetric analysis, let us assume that the following figures are obtained in a Kjeldahl determination of total nitrogen in urine, the ammonia being determined by titration:

Three cubic centimeters of urine measured from a pipette with an error of 0.003 cc., or 0.1 per cent.

Twenty-five cubic centimeters of 0.1 N acid, measured from a burette with an error of 0.05 cc. To this must be added 0.025 cc. of possible error due to the 1 part per 1000 error in standardization, making the total error 0.075 cc. of 0.1 N solution.

Ten cubic centimeters of 0.1 N alkali used in back titration, with an error of measurements of 0.05 cc. from the burette. To this is added 0.01 cc. of error from the 1 part per 1000 error in standardization of the alkali, making the total 0.060 cc. of 0.1 N solution involved in measurement of the alkali.

To these we add the end point error of 0.05 cc.

The total error involved in the titration is therefore $0.075 + 0.060 + 0.050 = 0.185$ cc. which is 1.2 per cent of the 15 cc. of 0.1 N ammonia found by the analysis.

To this is added 0.1 per cent error in pipetting the urine sample.

The total possible error is therefore 1.3 per cent, despite the fact that all apparatus and solutions meet the requirement of 0.1 per cent accuracy.

The average probable error would, of course, be less, because some of the errors might be less than 1:1000, and not all would usually be in one direction.

The total possible error calculated above does not include those errors which may have occurred in the preliminary processes of digestion and distillation by which the nitrogen was transformed into ammonia and prepared for titration.

The Kjeldahl titration, in which the ammonia is calculated from the difference between the volumes of two standard solutions used, exemplifies a *titration by difference.* Because of the errors which accumulate upon the difference determined, it is difficult without unusual precautions to keep the possible errors of such titrations below 1 per cent.

An example of a simpler volumetric analysis by *direct titration,* is afforded by the titration of oxalic acid, which is used frequently in determining calcium oxalate. In this analysis there is only one measurement; standard permanganate is run into the oxalic acid solution until the end point is obtained. In such a titration, requiring 25 cc. of 0.1 N permanganate, the error, with the same limits of accuracy for burette and standard solution assumed in the preceding example would be 0.05 cc. for the burette, plus 0.025 cc. for the permanganate standardization, plus 0.05 cc. for the end point, or a total of 0.125 cc., which represents only 0.5 per cent of the amount of substance determined. By doubling the amount of the oxalate sample, and therefore of the standard permanganate required, the error would be reduced to 0.3 per cent.

COLORIMETRY

A colorimeter is a device by which the intensity of a given color in two solutions can be compared. If the intensity of color is quantitatively related to the concentration of a substance which it is desired to measure, colorimetry may be employed for quantitative analysis of that substance.

Several different types of procedure are utilized in colorimetry:

1. A series of solutions of different known concentrations, with correspondingly different coloration, are prepared in test tubes. With these the unknown is compared. The tubes are usually placed for comparison side by side in a holder called a comparator.

2. The unknown solution is diluted until its color matches that of a solution of known strength. This is spoken of as dilution colorimetry.

3. Light from the same source is passed through layers of known depth of both solutions. The depth of fluid in one or both is altered until the intensities of color in light transmitted through the unknown and standard

are equal. This is the type of comparison colorimetry used in most modern methods.

Other principles have been employed but have not been widely adopted. *Series of tubes with comparators* are used chiefly when intensity of color is not proportional to the concentration of the substance which is to be measured, or when the presence of other coloring matter interferes with comparison in a colorimeter. In the usual comparator the solutions, in tubes of equal diameter, are viewed against a background which transmits through each light of the same nature and intensity. If the unknown contains extraneous coloring matter besides that which it is desired to measure, a compensating tube, filled with a solution containing the extraneous matter in the same concentration as the unknown, can be placed behind the standard. The practical application of a comparator is described in connection with the colorimetric determination of pH.

Dilution colorimetry alone is little used except for relatively rough analyses; e.g., in some clinical hemoglobin estimations. It may, however, be combined to advantage with comparison colorimetry in certain types of analytical procedures.

Comparison colorimetry

Construction of usual type of comparison colorimeter. The general characteristics of colorimetric instruments are illustrated in the diagrammatic sketch of the Hastings bicolorimetric Duboscq instrument, in figure 88 of the chapter on the determination of pH. Light from a source of uniform intensity is transmitted, by means of reflectors and prisms, in parallel rays through the two fluids to be compared. By mechanical devices the depth of one or both columns of fluid through which the light passes can be regulated at will. The depths are so adjusted that the colors from the two solutions, which are projected into adjoining portions of a visible field, appear equal.

In modern designs of the standard type of apparatus, represented by the Duboscq, Kober-Klett, and similar colorimeters, adjustment of the depth of fluid is effected by moving the cups which contain the solutions. Into each solution dips a stationary glass plunger, which transmits upwards the light that comes to it through the colored solution below.

The cups consist of thick-walled cylinders of glass. The bottom of each cup is formed by a plate of clear glass. In some cups the bottoms are attached by means of cement, which is likely to be soluble in certain solvents. It is now possible and preferable to use cups with bottoms fused on or held in place by pressure without cement.

Millimeter scales with verniers permit measurement of the distance be-

tween the lower surfaces of the plungers and the bottoms of the cups to within 0.1 mm.

The field of the instrument, as viewed through the observing microscope is a circle divided into two halves. The dividing line is formed by adjacent ends of the rhombohedral prisms which transmit the light that has passed through the two cups and plungers. The prisms are so arranged that the light which illuminates the right side of the field has passed through the solution in the left cup, and vice versa. When the two halves of the field show identical color intensity, the concentrations of the solutions in the two cups approximate inverse proportionality to the depths of solution layer. The inverse proportionality between color intensity and layer depth does not, however, usually hold with exactness if the concentration of colored substance in one cup is more than 1.5 times that in the other. Colorimetry is accurate only when the unknown and standard do not differ by more than this ratio, and for the most accurate results the difference should be less.

The source of light

Care must be taken that the illumination of the two fields is equal. Equal illumination can be secured best by using an even, ample source of light, preferably diffuse daylight or white artificial light. There are numerous satisfactory lamps on the market. The one devised by Cullen is especially practical because it is adapted to use with comparators as well as colorimeters and can be improvised from stock electric fixtures. It can also be secured from American dealers in laboratory supplies. Light from a 150 to 300-watt Mazda lamp passes down through a filter of "daylight glass" into a sheet iron box about 12 x 12 x 12 inches in size. In the box the light strikes a reflector consisting of a white piece of sheet iron fastened at an angle of 45 degrees, from which the rays are reflected horizontally out through a slit about 4 inches high, formed by cutting away the lower part of one side of the box. The outside of the box is painted black, the inside a smooth, flat white. The 45-degree plate, illuminated from above, affords an ample smooth white source of diffuse light. The colorimeter is placed in front of the opening.

The Kober-Klett colorimeter is provided with a light in its base, which obviates the necessity of an outside source.

Testing the colorimeter

Before a colorimeter is used and at frequent intervals it should be tested with regard to the readings of the zero points on the two scales, equality of illumination of the two fields, and equality of transmitted color when equal layers of the same solution are received in the two cups.

The zero points. The cups, entirely empty, are first placed in position on the apparatus and carefully raised until the plungers come into contact with the bottoms of the cups. Then the readings on both scales should be zero. If this is not the case readings must be corrected for the zero point error. For example, if the reading on the left hand scale is +0.2 mm. and that on the right hand scale is −0.2, 0.2 mm. must be subtracted from all subsequent readings on the left hand scale and added to those on the right hand scale. The need for such corrections is obviated on some colorimeters by the introduction of devices by which the scales may be set at the zero points.

Equality of illumination. When the colorimeter is properly placed before the source of light both sides of the field should be equally bright and have the same appearance. If this does not occur either the source of light is improper or there is some defect in the optical apparatus. The commonest cause of such difficulties is injury to the mirror of the colorimeter, brought about by the inadvert spilling of chemical solutions, especially alkaline solutions.

Equality of transmitted color from both cups. Portions of the same colored fluid, usually the colorimetric standard solution, are placed in both cups. The cups are raised to the zero point to expel air bubbles that may have formed below the plungers. The depth of fluid in one cup is set at a suitable point, and a series of readings is made after the other cup has been repeatedly adjusted to make the two sides of the field look alike. The readings on the two scales, corrected for differences in zero points, should be identical.

Technique of colorimetric determination

The cups should never be so full that liquid is driven over the top when the cups are raised to the zero point. However, there should always be enough fluid to cover the lower ends of the plungers within the practical range of motion of the cups.

The standard solution is placed in one of the cups and the "unknown" in the other. When the cup receiving either solution is wet with a different one, both cup and plunger are rinsed with the new solution by placing some of this in the cup which is moved up and down to wash the plunger. The rinsing solution is then discarded, and the portion to be analyzed is placed in the cup. The depth of layer of the standard is set at some convenient point, usually 15 or 20 mm., and the height of the cup containing the unknown is adjusted until the color intensities of the two fields match. Several such adjustments are made in succession, and the average of the readings obtained is used for the calculation.

Calculation. Within certain ranges the intensity of color is directly proportional to the concentration of the coloring substance and inversely proportional to the depth of the column of fluid as measured on the colorimeter scale. Therefore, if C_u and C_s represent concentrations, and U and S represent depths of layer for the unknown and standard, respectively,

$$C_u = C_s \times \frac{S}{U}$$

Limits of accuracy and sources of error. Few observers can, with the usual colorimetric instruments, match colors with an error of less than 1 per cent; for most analysts the error is probably nearer 2 or 3 per cent. The accuracy is greatly influenced by characteristics of the individual. Some persons are incapable of matching colors; others have difficulties with certain special colors. Adjustments should be made rapidly to avoid tiring the eyes.

The *range* within which depth of solution is proportional to intensity of color is quite limited. Standard and unknown can seldom be permitted to differ from one another by more than about 50 per cent, i.e.

$$\frac{S}{U}$$

must lie between 0.7 and 1.5. If the difference is greater than this another standard must be used or the test must be repeated with a different concentration of the unknown.

In some analyses, after the colors have been developed either the standard or unknown may be diluted so that the two colors are sufficiently close for comparison (e.g., see Benedict's blood sugar method). Such a *combination of dilution and comparison colorimetry* is probably feasible whenever the color is due purely to a single substance. It can not, however, be used in all methods.

Correction curves, indicating the deviation of observed $S:U$ ratios from exact inverse proportionality to concentrations, may be used when it is desirable to compare solutions so different in concentration that the rule of inverse proportionality does not hold with exactness. To construct such a curve, solutions of varying known strengths are compared with a single constant standard, and the readings are plotted on coordinate paper.

Sometimes there is lack of exact proportionality between color intensity and concentration, even when the $S:U$ ratio is near unity. In this case the cause is usually the presence of interfering colors other than the one that is being measured. The most extreme case is, of course, that in which the

reaction involves transformation from one color to another, as in pH determinations or the Folin colorimetric ferricyanide blood sugar procedure. Under these circumstances the range within which comparison colorimetry is practicable is extremely limited and can not be enlarged by dilution methods or by correction curves, because the color change affects quality of color as well as intensity. If the changes involve quantitative change from one colored substance to another, as in the change of an indicator from the acid to the alkaline form, a *bicolorimeter* may be used (figure 88), in which the light passes through measured depths of *two* standard solutions, one of each color. In other instances light filters which screen out the interfering color may be employed, as in the Folin sugar method.

Other colorimeters. Although the type of colorimeter represented by the Duboscq and Kober (9) instruments has been described as the standard instrument, other ones are available. Among these may be mentioned the Bock-Benedict (2) apparatus, in which the expensive rhombohedral prisms of the Duboscq instruments are replaced by mirrors. The Autenrieth and Koenigsberger colorimeter has been widely employed for less accurate determinations, especially in clinical work. The standard solution is carried in a wedge-shaped retainer in juxtaposition to a small standard cell containing the unknown. The two are viewed through a small slit. By moving the wedge the depth of the standard solution may be varied until its color equals that of the unknown. Myers (10) has employed the same principle in the development of a bicolorimeter. He uses two wedges with their bases in opposite directions to hold the two color stadards, and a third wedge for blank solution. The instrument is equipped with Duboscq prisms and eye piece and other improvements, and is built of metal, whereas the original Autenrieth apparatus is built of wood.

NEPHELOMETRY

A nephelometer is an instrument designed to measure the concentration of suspensions. The instruments are similar to colorimeters. The light, however, instead of passing through the solution in the plane of vision, passes into the solution at right angles to the plane of vision. The rays which strike particles suspended in the liquid are reflected into the field of vision. The brightness of the field is therefore proportional to the number of particles reflecting light.

Nephelometer attachments are made for Duboscq colorimeters. The plungers are replaced by tubes of uniform size to receive the fluids that are to be compared; while opaque cups of metal are substituted for the glass colorimeter cups. The portions of the tubes not blanketed by the cups are

illuminated by horizontal light. As the cups are moved up and down, the length of tube which is illuminated varies.

The Kober (9) instrument can also be converted into a nephelometer.

In the technique of its use the nephelometer does not differ greatly from the colorimeter, and determinations are carried out in the same manner in both types of apparatus. All light except the horizontal rays from a single uniform source must be excluded. Therefore it is necessary for both the nephelometer and the light source to be housed together in a special case of some kind that shuts out extraneous light rays.

The unknown and standard may differ somewhat in concentration, but the nature of the suspended matter, especially its degree of disperson in the two suspensions, must be identical, and there must be no tendency to settling during the determination. To insure such uniformity and stability of suspensions is a matter of such difficulty that most analysts, including the authors, prefer to avoid the use of the nephelometer when other means of analysis can be applied. Bloor, Kober (9), Kleinman (8) and Denis have used it most extensively for biological work. Bloor has recently abandoned the nephelometric technique for blood fat determinations and adopted the oxidation procedure of Bang (see lipoid chapter).

Determination of suspended matter by the light absorbed (turbidity) in an unmodified colorimeter has been practiced, but it appears to be even less satisfactory than nephelometry.

SPECIFIC GRAVITY DETERMINATIONS

The use of *specific gravity bulbs*, or hydrometers, such as are commonly used for urine specific gravities, is too familiar to require discussion. They provide the simplest means of gravity determinations when plenty of fluid is available and accuracy of ± 0.001 unit is sufficient. The only special precaution required is to keep the temperature of water solutions within 2°, and of alcohol within 0.5°, of the temperature for which the bulb is standardized.

For more exact gravity determinations *pycnometers* or specific gravity bottles are used. These are small stoppered flasks, or other forms of vessels, of which a sufficient variety for convenient use under varying conditions can be seen in any apparatus catalog. They are all used in the same general manner, to compare the weight of a given volume of a fluid either, (1) with its volume to determine density or absolute specific gravity, or, (2) with the weight of water at the same temperature to determine relative specific gravity.

Absolute specific gravities, or densities are expressed by the symbol $D\frac{t}{4}$,

indicating that the figure shows the density of the fluid compared with that of water at 4 degrees.

To determine absolute specific gravity the volume held by a pycnometer is first ascertained by weighing the water which it contains, and multiplying this weight in grams by the volume of 1 gram water at the observed temperature, e.g., by 1.0028 if the temperature is 20° (see table 2). The density $D\frac{t}{4}$ is the grams of liquid which the pycnometer holds divided by the volume in cubic centimeters ascertained in the above manner.

Relative specific gravities express the ratio of weight of observed fluid to that of water at the same temperature. Relative specific gravity is symbolized as $D\frac{t}{t}$, where t represents the temperature of both fluids, e.g. $D\frac{20}{20}$ indicates the density of a fluid compared with that of water when both are at 20 degrees. Urine specific gravity bulbs are usually calibrated to give relative specific gravity.

Effect of temperature. For water and dilute water solutions the change in specific gravity is about 0.0002 unit in density per degree centigrade. For many organic liquids the coefficient of expansion is greater; for alcohol it is 0.0008, or 4 times as great as for water. When a specific gravity is taken for comparison with standard values the temperature of the fluid must be fixed with an accuracy proportional to that which one seeks to obtain in the gravity determination. With urine a temperature rise of 5 degrees C. will change the gravity by 1 point in the third decimal place, e.g., from 1.021 to 1.020. Since the gravity of urine is usually measured only to the third place, fixation of the temperature within 2°C. of the standard temperature (e.g., between 22° and 18° if the standard is 20°) is sufficient to prevent significant error from temperature variation. If accuracy to the fourth decimal place is sought, it is necessary to keep the temperature within 0.2° of standard, or to make a correction for the temperature effect.

When specific gravities are desired accurate to more than 0.0002 of a unit, which is the change caused in water by 1 degree of temperature, a pycnometer with an open capillary outlet is filled with solution at a temperature slightly below the standard, and is then immersed in a bath at the standard temperature. As the fluid warms it rises through the capillary. When expansion has been completed the exuded fluid is removed, and the pycnometer is weighed.

It is preferable to take such gravities with the pycnometer at tempera-

TABLE 10

RELATIVE PROPORTIONS OF ALCOHOL BY WEIGHT AND VOLUME AND SPECIFIC GRAVITY OF ALCOHOL-WATER MIXTURES AT 20°

PER CENT ALCOHOL BY VOLUME	PER CENT ALCOHOL BY WEIGHT	SPECIFIC GRAVITY $D\frac{20}{4}$	PER CENT ALCOHOL BY VOLUME	PER CENT ALCOHOL BY WEIGHT	SPECIFIC GRAVITY $D\frac{20}{4}$
1	0.795	0.9967	42	35.150	0.9417
2	1.593	0.9953	43	36.050	0.9430
3	2.392	0.9938	44	36.955	0.9412
4	3.194	0.9924	45	37.865	0.9385
5	3.998	0.9910	46	38.778	0.9376
6	4.804	0.9897	47	39.697	0.9358
7	5.612	0.9884	48	40.622	0.9339
8	6.422	0.9872	49	41.551	0.9320
9	7.234	0.9860	50	42.487	0.9301
10	8.047	0.9847	51	43.428	0.9281
11	8.862	0.9835	52	44.374	0.9261
12	9.679	0.9823	53	45.326	0.9240
13	10.497	0.9812	54	46.283	0.9220
14	11.317	0.9801	55	47.245	0.9199
15	12.138	0.9789	56	48.214	0.9178
16	12.961	0.9778	57	49.187	0.9156
17	13.786	0.9767	58	50.167	0.9135
18	14.612	0.9756	59	51.154	0.9113
19	15.440	0.9746	60	52.147	0.9090
20	16.269	0.9735	61	53.146	0.9068
21	17.100	0.9725	62	54.152	0.9045
22	17.933	0.9714	63	55.165	0.9022
23	18.768	0.9703	64	56.184	0.8999
24	19.604	0.9692	65	57.208	0.8976
25	20.443	0.9680	66	58.241	0.8952
26	21.285	0.9670	67	59.279	0.8928
27	22.127	0.9657	68	60.325	0.8904
28	22.973	0.9646	69	61.379	0.8879
29	23.820	0.9634	70	62.441	0.8855
30	24.670	0.9622	71	63.511	0.8830
31	25.524	0.9609	72	64.588	0.8805
32	26.382	0.9596	73	65.674	0.8779
33	27.242	0.9583	74	66.768	0.8753
34	28.104	0.9569	75	67.870	0.8727
35	28.971	0.9555	76	68.982	0.8701
36	29.842	0.9541	77	70.102	0.8674
37	30.717	0.9526	78	71.234	0.8647
38	31.596	0.9511	79	72.375	0.8620
39	32.478	0.9495	80	73.526	0.8592
40	33.364	0.9479	81	74.686	0.8564
41	34.254	0.9463	82	75.858	0.8536

TABLE 10—*Concluded*

PER CENT ALCOHOL BY VOLUME	PER CENT ALCOHOL BY WEIGHT	SPECIFIC GRAVITY $D\frac{20}{4}$	PER CENT ALCOHOL BY VOLUME	PER CENT ALCOHOL BY WEIGHT	SPECIFIC GRAVITY $D\frac{20}{4}$
83	77.039	0.8507	92	88.310	0.8224
84	78.233	0.8478	93	89.652	0.8189
85	79.441	0.8448	94	91.025	0.8152
86	80.662	0.8418	95	92.423	0.8114
87	81.897	0.8387	96	93.851	0.8075
88	83.144	0.8356	97	95.315	0.8033
89	84.408	0.8325	98	96.820	0.7990
90	85.689	0.8292	99	98.381	0.7943
91	86.989	0.8259	100	100.000	0.7893

tures as warm as or warmer than that of the room air for two reasons. (1) It is better after removal of the pycnometer from the bath to have the fluid contract and draw back down the capillary than to have it expand further, and perhaps ooze out, before the weighing is finished. (2) If a glass vessel containing fluid colder than the air is weighed, moisture from the air may condense on the glass. Undesirable haste in weighing may be necessary to prevent error from one or both of these causes if the pycnometer is filled much below room temperature.

In place of bringing a water solution in the pycnometer to standard temperature, one may fill at any room temperature within a few degrees of the standard, and ***correct the result for temperature*** by adding 0.0002 to the observed gravity for each degree centigrade above standard at which the weighing is made, or subtracting 0.0002 from the observed gravity for each degree below standard. Such correction, if for a temperature difference of less than 5° is accurate within 0.0001 unit of specific gravity. For an example of its application, and of special precautions necessary for accuracy with a micro-pycnometer, see section on plasma specific gravity in chapter XXI.

Besides urine and plasma, one is most likely to use density determinations to ascertain alcohol concentrations in water-alcohol solutions. In table 10 are given the densities of water-alcohol mixtures.

CLEANING MERCURY

Mercury should not be allowed to come into contact with other metals, because of its tendency to form amalgams. Mercury that does become contaminated in this manner must not be returned to the stock supply until

it has been freed from amalgam. Such contaminated mercury leaves adherent films on glass.

Slightly contaminated mercury is placed in a beaker or evaporating dish to expose a large surface. The surface is covered with a mixture of equal volumes of concentrated nitric acid and water, which is allowed to stand for a few minutes. This dissolves the major part of the scum from the surface of the mercury. The acid is then washed off by decantation with water. The mercury is next poured in a fine stream through a cleaning tower filled with dilute nitric acid (a 10 volume per cent solution of concentrated nitric acid). The tower consists of a glass tube about 1 meter high and 25 mm. in diameter. At the bottom a tube of about 4 mm. bore, curved in the form of a goose-neck, is fused or attached by rubber stopper. Mercury from a funnel drawn out to a fine capillary falls in a fine stream into the nitric acid in this tower, collects in the bottom under the acid, and eventually passes out through the curved tube at the bottom. The mercury thus washed is then poured through a second tower filled with water in order to wash off the major portion of the acid. It is finally washed repeatedly with distilled water by decantation in an open dish or beaker until the washings are neutral.

Mercury which contains large amounts of other metals is best purified by distillation *in vacuo*. Amalgams can, however, usually be eliminated by oxidation. The mercury is covered with a thick layer of equal parts of water and a saturated solution of sodium hydroxide in a wash bottle attached to a suction tube. A current of air is bubbled through the mercury and alkali for several days, the alkali being renewed from time to time as it becomes soiled. When aeration is completed the mercury is subjected to the usual washing procedure described above.

Gross contaminations of grease and dirt can be removed by straining mercury through towels.

ETCHING GLASS APPARATUS

To etch new calibration marks, corrections, etc., on glass apparatus, warm the apparatus by passing it through a flame a few times and then paint it with a thin, even layer of melted paraffin. The layer remains more even if the vessel is rolled while the paraffin hardens. The desired marks are then cut through the paraffin with a sharp point. A sharp pencil will serve the purpose. The sharp point should be held in a fixed position while the flask, burette or pipette is rotated against it. The marks are then painted with hydrofluoric acid by means of a long handled brush. ***If hydrofluoric acid comes in contact with the***

hands it may cause subsequent necrosis. After the acid has acted for about twenty minutes, it is washed off under the tap. The vessel is then warmed until the paraffin melts, when the latter is wiped off with a towel. If it is desired that the marks stand out more clearly, they may be filled with the colored wax of a wax pencil or with black asphalt paint. The coloring substance is smeared on warm and then lightly wiped away from the surface, leaving only that which was caught in the etched lines. If a new mark is placed on a vessel previously calibrated, the correct mark is indicated by some distinguishing sign, such as a short arrow.

LUBRICATION OF GLASS STOP-COCKS

Success in handling any apparatus that involves the use of stop-cocks, particularly apparatus for measurement of gases, depends to no small extent upon proper lubrication of these stop-cocks. This, in turn, depends upon the careful application of satisfactory lubricants.

Vaseline-rubber lubricant.[6] One part of pure, non-vulcanized, raw rubber gum is cut into small pieces and dissolved in 4 parts of vaseline and 1 part of paraffin. The mixture is heated in an oven at 110°, with occasional stirring, until the rubber has disintegrated and mass has become apparently uniform. This requires about two days. Then the mixture is heated with a micro burner for a half-hour at 150° to 160°, and is strained through cotton gauze to remove any undissolved particles.

This lubricant is used together with vaseline. A thin layer of vaseline is first applied to the core of the stop-cock, and then a thin layer of the vaseline-rubber lubricant. If the room temperature is high more of the rubber lubricant and less of the pure vaseline is used, and vice versa. This combination, in the experience of the authors, yields joints that hold more reliably and require lubrication less frequently than any other mode of lubrication they have attempted.

The stop-cock with its adjoining connections must be scrupulously cleaned before it is lubricated. Commercial pipe cleaners are useful in cleaning connections and bores. Old grease may be removed by scrubbing with a little ether.

Lubricant which enters the bore of the stop-cock and the adjoining connections must be removed. Such entrance can be minimized by applying only ***a minimal amount of grease*** in a ring about each end of the stop-cock and working this in by turning the core in its casing. A well lubricated cock should appear transparent throughout and should turn with perfect ease.

[6] This lubricant can be obtained from Eimer and Amend, New York.

Pressure should not be exerted upon a stop-cock either during the process of lubrication or in the subsequent use of the apparatus. Such pressure tends to drive out the lubricant and to wear the cock. If bits of lint or other foreign matter are left between the core and casing during lubrication they will etch the cock, leaving channels with resultant leaks.

RUBBER STOPPERS AND CONNECTING TUBING

Only the highest grade tubing should be used for stoppers and for connecting tubes which may come into contact with reagents or reaction mixtures. Such tubing and stoppers, when purchased, are often covered with talc, sulfur "bloom," or other surface impurity which must be removed, together with any soluble matter, before the rubber can be used.

Cleaning rubber. For ordinary purposes stoppers can be sufficiently cleaned by scrubbing in water, but it is usually desirable in addition to treat them with alkali in the manner prescribed for rubber tubing below.

Tubing is first scrubbed by drawing through it a wire to which a wet cloth has been attached. This is repeated until all obvious "bloom" has been removed. The tubing is then boiled for five minutes in dilute sodium hydroxide (about 0.5 normal). The alkali is washed off with running water. The tubing is again boiled, this time in dilute hydrochloric acid (1 volume of concentrated hydrochloric acid to 25 of water), for five minutes. It is then washed with tap water until the washings are neutral. Tubing may be best washed by attaching it to a faucet.

Preserving rubber. Tubing does not keep so well after the "bloom" has been removed; therefore only enough should be prepared for immediate needs. Rubber goods tend to deteriorate with time, becoming non-elastic and cracking. Deterioration is accelerated by heat and drying. It is well, therefore, to keep all tubing in a cool place, best of all in a sealed desiccator containing an open bottle of turpentine. These precautions are especially important in hot climates and summer weather.

Only higher grade black rubber tubing, which has been cleaned in the manner described above, should ever be used in the administration of intravenous solutions.

EYE PROTECTION

Protective glasses should be worn whenever an operation is carried out that may result in the explosion or collapse of glass vessels, or in the spurting or spattering of alkaline or hot acid solutions. Distillations *in vacuo*, and the acid digestions and alkaline distillations connected with Kjeldahl

analyses call for such protection. Even *minor* explosions or spatterings of caustic solution may result in complete destruction of eyesight. The writer has known two friends and colleagues who have thus been blinded for life, one by glass splinters, the other by a few drops of alkali. The best practice is to have a comfortable pair of ordinary spectacles with fairly large lenses, or plain glass plates, and *wear them as a matter of regular routine always while in the laboratory*. The putting on of special protective goggles at times when protection seems necessary is a much less sure practice, because the damaging accident is likely to occur at a moment when no danger is expected.

CHOICE OF METHODS

In a given case the relative desirabilities of the different methods available for an analysis depend upon conditions, such as the facilities of the laboratory, the number of determinations to be done, and the personal characteristics of the analyst. If only one or a few analyses are to be made, it may be simpler to do them gravimetrically than to make standard solutions and check the technique for a titration or colorimetric procedure, even when the latter would be preferable for numerous routine determinations. When the laboratory possesses one of the modern rapid-weighing balances, and the porous Jena glass crucibles can be used, gravimetric determinations may be so convenient and simple that they are preferable even for routine.

In choosing between colorimetric and titration methods both the laboratory equipment and the physiological equipment of the analyst play rôles.

Some colorimeters give fields more capable of exact comparison than do other instruments even of the same make. Furthermore some persons have eyes better adapted to colorimetric work than others: there are variations both in degree of sensitivity to color intensities and in the rate at which the eye becomes fatigued. In consequence one analyst may rejoice in a given colorimetric method and another may condemn it.

For one to whom the manipulation of precise measuring apparatus is attractive, the gasometric methods combine the rapidity of titration or colorimetric procedures with the advantages of gravimetric analyses in objective accuracy and freedom from dependence on standard solutions.

BIBLIOGRAPHY

1. Bang, I.: Micromethoden zur Blutuntersuchung. München, 1920.
2. Bock, J. C., and Benedict, S. R.: A new form of colorimeter. *J. Biol. Chem.*, 1918, 35, 227.
3. Bonner, W. D., and Branting, B. F.: The composition of hydrochloric acid of constant boiling point. *J. Am. Ch. Soc.*, 1926, 48, 3093.

4. Design and test of standards of mass. U. S. Bureau of Standards, Circular, No. 3. U. S. Gov't Printing Office, 1918.
5. FOULK, C. W., AND HOLLINGSWORTH, M.: A precision measurement of the composition of the constant boiling mixture of hydrogen chloride and water. *J. Am. Ch. Soc.*, 1923, **45**, 1220.
6. HULETT, G. A., AND BONNER, W. D.: A method for preparing standard hydrochloric acid solutions. *J. Am. Chem. Soc.*, 1909, **31**, 390.
7. HULETT, G. A.: Über Kalibrierung einer Glasröhre und einige Kompressibilitätskoëffizienten. *Z. physik. Chem.*, 1900, **33**, 237.
8. KLEINMANN, H.: Zur Nephelometerapparatur. *Biochem. Z.*, 1926, **179**, 301.
   Weitere Beiträge zur Nephelmeterapparatur und der Methodik nephelometrischen Arbeitens. *Ibid.*, 1923, **137**, 144.
9. KOBER, P. A.: An improved nephelometer-colorimeter. *J. Biol. Chem.*, 1917, **29**, 155.
   Further improvements in the nephelometer-colorimeter. *Ibid.*, 1921, **47**, 19.
10. MYERS, V. C.: A colorimeter for bicolorimetric work. *J. Biol. Chem.*, 1922, **54**, 675.
11. OSBORNE, N. S., AND VEAZEY, B. H.: The testing of glass volumetric apparatus. U. S. Bureau of Standards, Scientific papers, No. 92, U. S. Gov't Printing Office, 1908.
12. REHBERG, P. B.: A method of microtitration. *Biochem. J.*, 1925, **19**, 270.
13. SHOHL, A. T.: A pipet for micro-analyses. *J. Am. Chem. Soc.*, 1928, **50**, 417.
14. SÖRENSEN, S. P. L.: Recherches titrimétriques. II. Sur l'examen de l'oxalate sodique et sur son emploi dans les dosages volumétriques. *Compt. rend. d. Lab. Carlsberg.*, 1904, IX, 135.
15. TREVAN, J. W., AND BAINBRIDGE, H. W.: The estimation of calcium in blood serum. *Biochem. J.*, 1926, **20**, 423.

For detailed theoretical and practical discussions of colorimetry and nephelometry consult "Photometric Chemical Analysis" Volume I, Colorimetry; Volume II, Nephelometry, by John H. Yoe. Published 1928 and 1929, respectively, by Wiley and Sons, Inc., New York and London.

CHAPTER II

Special Biochemical Technique

The composition of blood and excreta is influenced *in vivo* by the activities of various bodily functions, and, furthermore may change rapidly *in vitro*. To control the conditions under which the material is collected and brought to analysis in such a manner that the results shall yield significant interpretations, special techniques have been developed, which will be discussed in the following pages.

PREPARATION OF SUBJECT

The compositions of the blood, other body fluids, and excreta are affected not only by pathological conditions, but also by physiological responses to various factors, such as diet, muscular and digestive activity, and even emotion. Such factors can cause blood or urine from healthy people to show in some constituents sudden changes from the usual normal values equaling the changes encountered in advanced disease; for example, less than a minute of severe exercise may lower the bicarbonate content of the blood as much as does fairly advanced diabetic acidosis. In order to interpret the results of clinical analyses it is therefore frequently necessary to fix or know the conditions of rest or activity, digestion or fast, and previous regime of the subject. The conditions that must be controlled or known depend upon the nature and object of the analyses undertaken. Consequently no general rule can be laid down: the person in charge of the examination should be familiar with the known physiological factors which may influence his results; factors that we have attempted to outline in the volume on "Interpretations." Only such familiarity can indicate for each type of determination the precautions which must be observed, and likewise those the observance of which is superfluous. As a general statement one can say little more than that blood should, for most analyses, be drawn during a period of more or less complete physical and emotional quiet, and that when sugar is to be determined the time of drawing must be definitely related to the time and nature of the preceding meals. Furthermore, the 24-hour output of most excretory substances, such as total nitrogen, urea, uric acid, and the mineral elements, can be interpreted only by comparison with food intake.

Whenever possible, and always when the result leads to an interpretation at variance with that yielded by other clinical data, or for any other reason appears open to doubt, it is desirable to repeat the examination with fresh material, obtaining a check not only upon the accuracy of the analysis but also upon the effect of physiological factors *in vivo* at the time the material is obtained.

DRAWING BLOOD FOR ANALYSIS

In the volume on "Interpretations" it is pointed out in several connections that blood is not uniform in composition throughout the circulation. Arterial blood differs from venous blood, which in turn varies from time to time and from place to place depending on the functional activity and the rate of circulation in the tissues through which it has passed. These variations are not without significance in certain types of studies. They may involve not only CO_2 and O_2, but also water and electrolytes, glucose and lactic acid.

Arterial puncture. **For certain purposes arterial blood is preferable to venous. It can be obtained by arterial puncture, as has been demonstrated by Hürter (21), Stadie (34) and others, from the radial, brachial or femoral arteries. A No. 20 gauge hypodermic needle sharpened to a fine edge with a short bevel (about 45 degrees) and attached to a syringe of suitable size, is used for such a puncture. The point of puncture is selected where the artery is superficial and easily palpable. The skin is anesthetized with a little novocaine. The needle is then passed through the skin at this point in the direction of the artery. Under the guidance of the fingers and the arterial pulsation the needle is passed through the arterial wall at an angle of about 45 to 60 degrees with the plane of the artery. When the needle has entered the arterial lumen the force of the blood pressure is sufficient to fill the syringe without suction. Occasionally the vessel contracts so violently for a short period after it has been entered that the blood flow is entirely shut off. After a moment, however, the vessel will dilate again and the blood will flow freely. When the needle is withdrawn firm pressure must be applied over the artery for a few minutes.**

Cutaneous blood. Blood which flows freely from a cutaneous puncture is, as far as chemical composition is concerned, indistinguishable from arterial blood; probably because after the first drops only the smaller arterial radicles continue to bleed (5, 14). Small amounts of blood for micro-analysis can therefore be secured by cutaneous puncture or incision when

arterialized blood is required. Drucker and Cullen (5) have drawn blood anaerobically from such incisions by immersing the part in sterile paraffin oil. Not more than 0.2 cc. of blood can be obtained easily from the ear or finger tip, although larger amounts may be collected from the heels of infants by freer incision (5). In any case the puncture or incision must be so large and deep that the necessary amount of blood can be secured without the production of stasis. The first drop that flows from the wound should be discarded.

Arterialized venous blood. Dautrebande, Davies and Meakins (3) have pointed out that the difference between venous and arterial blood drawn from the arm is greatly influenced by the temperature of the part. Cold slows down the circulation and magnifies the difference, while heat has the reverse effect. They found that blood drawn from the antecubital veins after the arm has been immersed for ten to twenty minutes in a bath at 45°, is almost indistinguishable in oxygen content from arterial blood. Goldschmidt and Light (15) find that blood from the veins on the back of the hand after similar treatment is even more nearly arterial, presumably because it has passed through less actively functioning tissue.

They immerse the hand and wrist in water at 45° to 47° for ten minutes. At the end of this time the hand is withdrawn far enough to permit the insertion of the needle into one of the veins. The heat distends the vessels so that they are easy to enter. A vein close to the knuckles is the most satisfactory. The needle is introduced with the point directed towards the fingers. Care must be taken not to pierce the opposite wall of the vein, and slight pressure must be applied after the needle is withdrawn.

Venous puncture. Even if arterial blood is not required, it is inadvisable to alter the composition of venous blood in the process of securing a sample. Before venous puncture the arm should be at least naturally warm and not chilled. The production of venous stasis should be avoided as far as possible. If a tourniquet is required it should be applied immediately before the vein is entered and removed as soon as the blood begins to flow. Slight momentary manual pressure just above the puncture will usually suffice to distend the vein.

ANAEROBIC DRAWING AND PRESERVATION OF BLOOD FOR GAS AND ELECTROLYTE DETERMINATIONS

A. The mercury sampling bulb (fig. 6). According to the experience of the authors, blood used for accurate determination of the gases, or of other

constituents which are affected by changes in the gases, must be drawn entirely without contact with air, or with oil or any other fluid thus far tested except mercury. For meeting these necessities the device shown

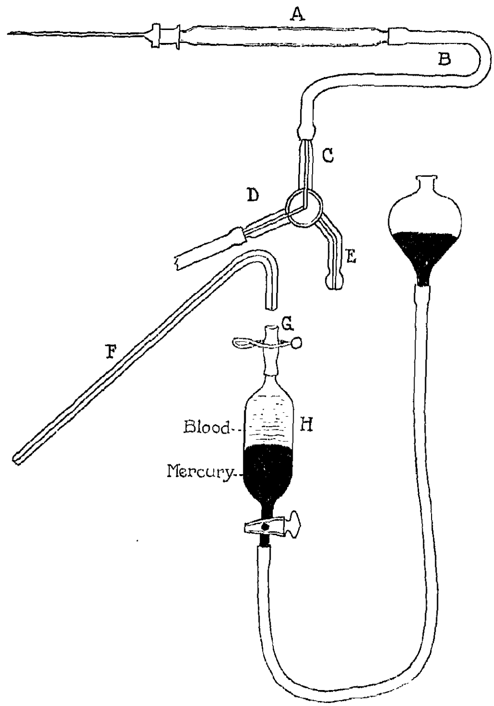


FIG. 6. Tube and needle for drawing blood without contact with either air or oil, and for preserving blood or plasma over mercury. *A* is a glass tube with one end ground to fit the needle. To draw blood *H* is first coated with oxalate. *G* and *E* are connected, and *H*, *G*, and *E* as far as the cock are filled with mercury. The leveling bulb is then lowered below *H*. *C* and *D* are connected and the needle is inserted into the vessel. Blood is drawn through the narrow-bore tube *B* into *D*. Then the cock is turned to connect *E* and *C* and the blood is drawn into *H*. *E* is then removed from *G*, the blood in *H* is mixed with oxalate by inverting *H* a few times. *H* is then immersed in ice water unless the blood can be analyzed immediately. *F* can be used to draw plasma or serum from a centrifuge tube into *H*.

in figure 6, introduced by Austin *et al* (1), has proved its practicability during a number of years in the laboratories of the authors and others. The sampling bulb is convenient, not only for drawing the blood, but also for

defibrinating it, and for chilling it by immediate immersion in ice water, which must be done routinely when oxygen or carbon dioxide determinations are to be carried out on the whole blood with maximal precision.

If the bulb with blood or serum is placed in a refrigerator the lower stop-cock should be left open and the leveling bulb elevated. Otherwise negative pressure, due to contraction of the bulb contents with fall of temperature, may cause formation of a small evacuated space into which gases pass from the blood.

The bulbs are also useful for the *removal from centrifuged blood of serum and plasma,* and for their preservation until analysis. For this purpose the capillary tip shown in figure 6 is used. It is connected with the sampling bulb and filled with mercury by means of the leveling bulb. The tip is then inserted beneath the surface of the serum or plasma, which is covered usually with oil. During the insertion care must be taken that no drop of mercury falls into the serum, or cells will be stirred up with the serum. With leveling bulb lowered, the serum or plasma is drawn into the sampling bulb.

Eisenman (7) has shown that the bulb can also be used for the *anaerobic defibrination* of blood. The blood, without anticoagulant, is discharged into the sampling bulb immediately after it is drawn. The bulb is then inverted repeatedly so that the small amount of mercury which remains in it is driven from one end to the other. As the blood coagulates the fibrin clot becomes intimately adherent to the mercury droplets. When clotting is complete the coagulum sinks to the bottom of the tube with the residual mercury leaving the defibrinated blood as supernatant fluid above the mercury.

B. The oil tube. A less desirable device, which enables one to draw the blood under a layer of paraffin oil, is shown in figure 7, I and III. Carbon dioxide will not to a significant extent escape from blood under oil in a tube that stands quietly for two or three hours, but is rapidly lost if the tube is agitated, as in centrifuging. Oil retards, but does not at all prevent, the escape of CO_2 from the surface of blood. The retardation is due to the interference with the movement of CO_2 molecules from the blood surface, and not to insolubility of the gas in the oil. In fact carbon dioxide is much more soluble in oil than in water (23).

Similarly oil retards only for a time diffusion of oxygen from air into the blood.

Another disadvantage of the oil tube is that when samples of whole blood are drawn by inserting a pipette through the oil into the blood it is almost impossible to get samples entirely free from oil droplets.

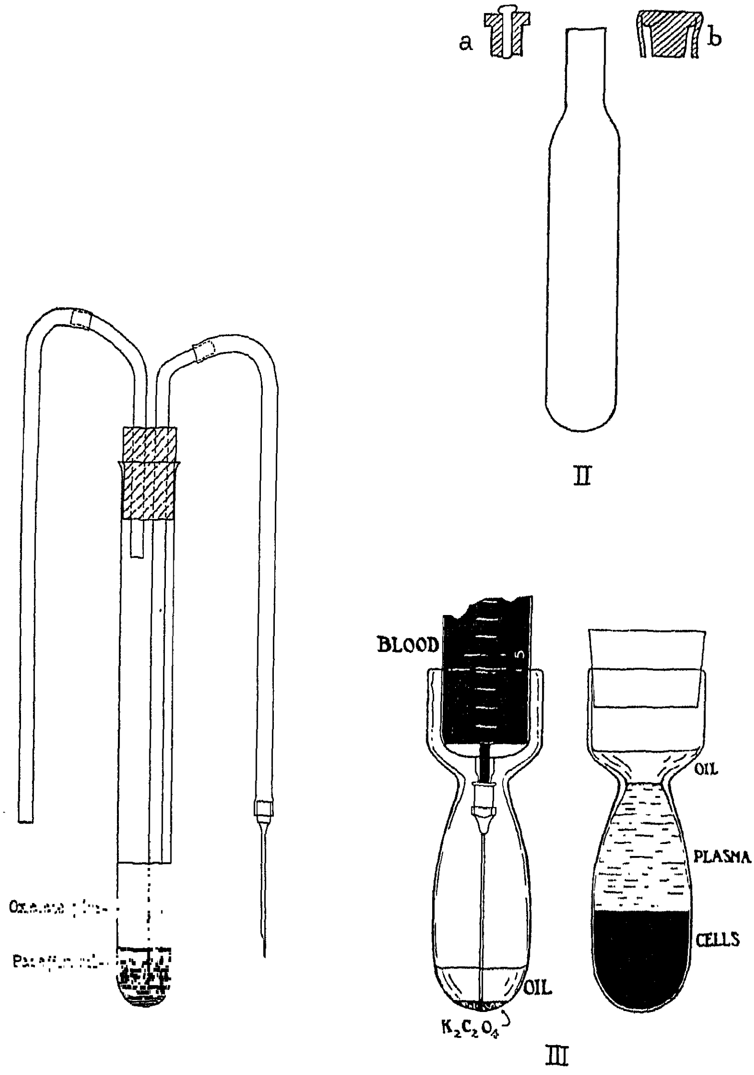


FIG. 7. Tubes for anaerobic collection and centrifugation of blood

I. Test tube for collecting blood under oil
II. Centrifuge tube to be completely filled with blood and stoppered
III. Constricted tubes of Myers and Muntwyler for centrifuging under oil

If the tube is to be centrifuged, or is to stand longer than two hours before samples are drawn for analysis, the oil should be replaced by a layer of paraffin. Some paraffin melting below 45° is heated just enough to melt it. The layer of paraffin oil on the blood is almost completely removed, and the melted paraffin is poured instantly over the blood in the tube. It mixes with the remaining oil and solidifies on cooling.

C. The oiled syringe. A third procedure is to draw the blood in a syringe that has been first filled with oil, of which all is ejected except the amount needed to displace air from the dead space near and in the needle. The barrel of the syringe can be coated with a film of oxalate to prevent coagulation. This method has the disadvantage that it mixes the blood with oil.

Blood drawn into an oiled syringe can be transferred to the mercury sampling tube described above. To do this the bulb *H* (fig. 6) and the rubber tube *G* above the bulb are completely filled with mercury. The needle is removed from the syringe into which blood has been drawn, and the oil is ejected while the syringe is held upright. The glass tip of the syringe is then inserted into rubber tube *H*, into which the tip should fit tightly. A small amount of mercury is displaced by the tip. The blood is then drawn into the sampling tube.

ANAEROBIC CENTRIFUGATION OF BLOOD

If serum or plasma is desired for determination of CO_2 or bicarbonate or chloride content, or for pH, centrifugation must be performed without contact with air. The centrifugation may be accomplished either in a stoppered tube which is completely filled with blood, so that no air bubbles are present under the stopper, or it can be done under a layer of oil in a constricted tube, or under a layer of paraffin. A layer of oil in an ordinary tube does not suffice to prevent loss of CO_2 and rise of pH during centrifugation.

For ***centrifuging in a completely filled tube*** a rubber stopper of the type shown in figure 7, II, *a* or 7, II, *b* is used. These tubes can be kept in various sizes suitable for different analytical purposes. The required amount of blood, drawn in a syringe in the manner described above, is injected through the needle beneath a layer of paraffin oil into a centrifuge tube small enough to be completely filled by the volume of blood available. The oil is displaced from the tube by the blood until only a thin layer remains at the top of the tube. A stopper of one of the two types shown is then inserted. Stopper *a* is a perforated rubber stopper, the lower part of which has been ground to fit the

tube, leaving a flange above to support it during the process of centrifugation. When the stopper is inserted the last vestiges of oil in the tube are displaced through the perforation, which is subsequently closed by the glass plug. Instead of this a "No-air" rubber stopper of the type used for vaccine bottles and illustrated in *b* may be used. A hypodermic needle is driven through the cap. When the stopper is inserted the oil is forced out through the needle, which is then withdrawn.

When the serum is to be removed the glass plug is removed from *c* and oil is introduced through the hole in the stopper as the latter withdrawn. With *b* the needle, this time attached to a syringe filled with oil, is driven through the cap and oil is injected as the cap is withdrawn. The serum is then transferred to a mercury sampling bulb as described above.

To ***centrifuge under oil*** the constricted tubes of Myers and Muntwyler (27*a*) may be used (fig. 7, III). The blood may be run from a syringe under a layer of oil into such a tube, which has been previously provided with oxalate, either powdered or, preferably, as a film coating the wall. The amount of blood is sufficient to bring the oil-blood interface to the constricted part of the tube. The surface of contact between the two fluids is thereby so diminished that during ordinary centrifugation no significant loss of CO_2 occurs from the plasma.

To ***centrifuge under solid paraffin*** the blood is run under a layer of paraffin oil in an ordinary centrifuge tube. The oil is then replaced by melted paraffin as described above on page 57. After the paraffin has hardened the tube is centrifuged. A hole is then made through the paraffin with a warm cork borer, and the plasma is drawn out through the opening, either into a pipette for immediate analysis, or into the mercury tube shown in figure 6, if the plasma is to be analyzed for CO_2 or pH later (see p. 815 of Van Slyke, Wu, and McLean (38)). For analyses of plasma other than of CO_2, $BHCO_3$, or pH, anaerobic preservation during the interval between centrifugation and analysis is unnecessary.

MEASURING BLOOD SAMPLES

To measure a sample from the mercury sampling bulb (fig. 6), the latter is inverted several times, with the stop-cock closed, so that the movement of the mercury will thoroughly mix the blood cells and plasma. The mercury bulb is then returned to the upright position shown in the figure, and the clamp is removed from the tube *G*. The leveling

bulb should be at the upper level shown in figure 6. As soon as the clamp is removed from the rubber outlet the tip of a pipette is inserted into the rubber tube. If the pipette tip is too narrow to fill the lumen of the rubber tube the pipette tip is surrounded with a rubber ring consisting of a section of narrow bore tubing about 1 cm. long. As soon as the pipette is in position the cock at the bottom of the sampling bulb is opened, and mercury pressure forces blood up into the pipette.

To measure a sample of blood from the oil tube (fig. 7, I or III), a pipette is closed at the upper end and the tip is thrust quickly through the oil layer. The sample is then drawn into the pipette by suction. It is difficult to carry out this manoeuvre without having a droplet of oil enter the pipette on top of the blood. It is consequently desirable to use a pipette that delivers between two marks rather than one that is calibrated for complete delivery. With the former the oil droplet remains in the pipette after the sample has been delivered for analysis, while with a pipette calibrated for complete delivery the oil would be delivered as part of the sample to be analyzed.

PREVENTION OF COAGULATION OF BLOOD

For the prevention of coagulation four methods have been generally employed: defibrination, and addition of oxalate, citrate, or heparin. Hirudin, which is the active principle of the secretion of the buccal glands of leeches, although widely employed in experimental work in former years, is now little seen. Preparations of high potency are hard to secure. It has now been quite replaced by heparin, apparently a compound of glycuronic acid introduced by Howell (20).

Defibrination is usually employed when mineral analyses are to be made on the separated serum, or when for any purpose it is desirable to avoid the introduction into the blood of foreign substances. The removal of fibrin can apparently be accomplished without appreciable alteration in other constituents of blood (7).

It is most easily carried out by stirring the blood in a tube or other cylindrical receptacle with a footed glass rod. The clot adheres to and can easily be removed with the rod. Care must be taken to keep the rod from coming into contact with the sides of the vessel, because this traumatizes cells, resulting in hemolysis. Defibrination of blood by stirring it in contact with air is unsatisfactory for many types of analysis because it permits loss of CO_2 and absorption of oxygen, which change not only the reaction and gas content of the blood but also the

anion and water distribution between cells and serum (see chloride chapter in "Interpretations" volume). The technique of defibrination described above can be performed under a layer of paraffine oil; but this does not eliminate CO_2 loss when the fluid is agitated by stirring.

The anaerobic defibrination technique of Eisenman (7), which is described above, is probably the most satisfactory method of preparing whole blood for electrolyte or other analyses without addition of anticoagulants.

If analysis of serum alone, and not of cells, is intended it is simplest to obtain the serum from blood which has been allowed to clot spontaneously under anaerobic conditions.

Potassium, sodium, or ammonium oxalate in a concentration of 0.1 to 0.2 per cent is an effective anticoagulant. Even in this low concentration, however, it causes slight shifts of water and electrolytes between cells and serum and a demonstrable alteration of the CO_2 combining capacity (6). The red blood cells always shrink somewhat. This results necessarily in a dilution of the non-diffusible components of the serum and a concentration of those in the cells with variable effects on Cl and HCO_3. It is obvious that *blood treated with oxalate can not be used if determination of calcium* is to be done, and that sodium and potassium oxalates prevent determination of Na and K and of total base. In greater concentration than 0.3 per cent oxalate causes serious alterations of the constitution of the blood, even frank hemolysis.

Potassium has been more used than other oxalates because it dissolves more readily.

Although the disturbing effects of oxalate on the distribution of water and electrolytes must be considered in accurate work, by proper care they can be reduced to limits that are negligible for many analyses, including CO_2 content and pH of plasma. This care consists of using minimal anticoagulant doses of a neutral purified preparation of the salt. Oxalate is likely to contain small amounts of carbonate, which give it an alkaline reaction. If whole blood is to be analyzed for sugar or nitrogenous components such oxalate may be used without affecting the results. If plasma CO_2 or pH is to be determined, the addition of alkaline oxalate is to be avoided.

Whole blood which is to be analyzed for sugar or nitrogenous constituents may be collected without special precautions in open bottles containing a small amount of powdered potassium oxalate. The blood is shaken at once to dissolve and mix the oxalate, and only enough salt is used to insure prevention of coagulation.

If plasma is to be removed, especially for electrolyte or gas determinations, the authors prefer the following technique. The potassium oxalate, unless neutral in reaction, should be recrystallized from hot water and washed with cold water. From the recrystallized salt a 30 per cent solution is prepared. This is colored with phenol red and brought to a pH of 7.4 ± 0.2 by the addition of potassium hydroxide or oxalic acid. The solution should be readjusted each time it is used. Enough of this solution is measured into the tube, syringe, or other blood receptacle to give a concentration of 3 mg. of oxalate per cc. in the blood (0.01 cc. of solution per cubic centimeter of blood). By rotating the vessel the oxalate is spread in a film on its walls. It is then dried in a stream of air. The tubes must not be heated to accelerate drying because this decomposes the oxalate and produces alkaline carbonate. When the blood is introduced it comes into contact with only a little oxalate at a time, which is immediately dissolved. This procedure insures uniform rapid mixing of anticoagulant without agitation, prevents the formation of small clots, and reduces the danger of the hemolysis which may occur when blood stands in contact with slowly dissolving larger crystals of oxalate. In tubes thus coated woth oxalate, blood can be collected under oil or over mercury- with exclusion of air contact (see section above on anaerobic treatment of blood).

Citrate. Potassium and sodium citrate are efficient anticoagulants, but for analytical purposes they have, compared with oxalate, the disadvantage that they cause greater disturbances in reaction and cell volume.

Heparin, obtained from dog's liver by Howell (20), has characteristics that should make it an ideal anticoagulant for chemical studies. It is, apparently, a carbohydrate compound, probably a glycuronate, containing a certain amount of sulfuric acid and calcium. It gains its effect by causing an increase in antithrombin when it is added to plasma or serum. In purified form it prevents coagulation of blood in a concentration as low as 1 mg. per 100 cc., without altering any known physical characteristic of the blood. Earlier unpurified specimens tested in the laboratory of one of the authors (6) were found to have acid properties and a distinct buffer value at the reaction of blood, and were therefore unsuitable for electrolyte or acid-base studies. Apparently the purified product has not yet been subjected to such tests. From the character of the compound it seems unlikely that the minute amounts required will appreciably influence the chemical composition of blood.

PREVENTION OF CHEMICAL CHANGES IN SHED BLOOD

The changes which may occur in shed blood can be classified as those due to loss of CO_2 and gain of oxygen by exposure to the air, those due to spontaneous chemical reactions occurring in the blood, and those due to bacterial action. The changes due to air exposure are prevented by drawing and handling the blood with anaerobic technique. The other changes can be prevented, for the time of a working day at least, by *chilling the freshly drawn blood to 0°* and keeping it at that temperature. However, for routine clinical analyses of many types this is not a convenient or necessary procedure, and other devices have been found which will prevent the changes interfering with specific analyses.

Of the spontaneous changes the following are important:

1. Glycolysis, or decomposition of glucose with formation of lactic acid. This phenomenon has been discussed in some detail in the carbohydrate chapter of Volume I, and will be considered again in the chapter on sugar determination in this volume. Besides its effect on the sugar and lactic acid contents of the blood, it has a marked influence on the pH and CO_2 tension, the blood becoming more acid and the CO_2 tension higher as the process advances (8).

2. Decomposition of nitrogenous substances with the production of ammonia and products of protein digestion.

3. Hydrolysis of cellular organic phosphates, yielding inorganic phosphate which diffuses into the plasma. Conditions which promote this reaction or reverse it are discussed in the chapter on phosphorus.

4. Alteration of the permeability of the red blood cell membranes, disturbing the normal unequal distribution of inorganic elements between the two phases of the blood.

5. Respiration of the cells, with formation of CO_2 and disappearance of O_2. This process is tremendously accelerated by hemolysis.

These changes occur with significant rapidity only in the presence of the cells. When the whole blood is to be analyzed the analysis must be begun before any of the changes have proceeded far enough to affect the result. And when serum or plasma is to be analyzed it should be separated from the cells as soon as possible (certainly within an hour) after the blood has been collected. If analysis or centrifugation can not be done within less than an hour, the freshly drawn blood should be chilled *in ice water* (chilling in the air of a refrigerator is too slow) and kept near 0°C. until it can be used.

For the preservation of whole blood for *sugar determination* a mixture of sodium fluoride and thymol is satisfactory (22, 30). The addition to blood, at the time it is drawn, of 10 mg. of sodium fluoride and 1 mg. of thymol

per cubic centimeter is said to keep it, even at incubator temperature, for two or three days, in such a state that the concentrations of glucose, total non-protein nitrogen and the individual non-protein nitrogenous components remain unchanged unless there is gross bacterial contamination (30). The mixture also serves as an anticoagulant. Stadie (personal communication) finds that ammonium fluoride is more efficient than the potassium salt in preventing glycolysis. Lax and Szirmai (24) recommend substituting for the thymol an equal concentration of mercuric chloride. Either NaF or $HgCl_2$, by retarding or inhibiting the action of urease, *interferes with the determination of urea by urease methods* (28).

If bacterial contamination is prevented sodium fluoride alone will serve as anticoagulant and preservative (4, 25, 29). Use of 10 mg. of sodium fluoride per cubic centimeter will preserve sterile blood for as much as ten days without change of sugar or nitrogenous constituents. However, 3 times as much will not preserve infected blood (29). Although sodium fluoride retards the action of urease, Roe, Irish and Boyd (29) claim that its effect can be abolished if the blood is diluted 7 to 10 times.

It is evident that blood which must be kept or transported before analysis should be drawn with the same bacteriological precautions as blood intended for blood cultures, and should be transferred to sterile containers for transportation or preservation. Blood thus treated is suitable for determinations of blood sugar and non-protein nitrogenous constituents, if it is preserved with fluoride or fluoride and thymol. It can not, however, be used for the determination of electrolytes. If such determinations are desired serum or plasma must be separated as soon as possible after the blood is withdrawn. The serum or plasma, in hard glass containers, sterile, can be kept for a long time without undergoing any electrolyte changes of importance, except as regards pH and CO_2 content. No satisfactory method has been devised for indefinitely preventing changes of CO_2 and pH although sterile separated serum or plasma may be kept in the refrigerator in the mercury sampling bulbs described above for as much as twenty-four hours without change in pH or CO_2, if bacterial contamination is avoided.

For prevention of changes in phosphatides, respiratory gases, and permeability no effective preservative has been found. One must start analysis quickly after the blood is drawn, or chill it to 0°.

PRECIPITATION AND REMOVAL OF PROTEINS OF BLOOD AND PLASMA

Not only in the determination of non-protein nitrogen, but also in many other analyses, it is essential as a preliminary step to remove from biological fluids the proteins with or without other intefering organic sub-

stances. The most common procedures for the elimination of protein involve precipitation of the protein and its removal by either centrifugation or filtration. Occasionally for special investigations, a protein-free solution of the blood crystalloids is obtained by dialysis or by pressure filtration through collodion, but these procedures are not often used for routine analyses.

The protein precipitants which have been employed and found useful for certain special purposes are too numerous to mention. They are chiefly acids, salts of heavy metals, alcohols, and other organic compounds. From all these precipitants a few have survived by virtue of certain properties which make them either generally useful or especially adapted to specific purposes.

Among those which have found application in modern blood analysis are colloidal ion, metaphosphoric acid, picric acid, methyl and ethyl alcohol, trichloroacetic acid, and tungstic acid. The comparative behaviors of these precipitants have been studied by Hiller and Van Slyke (19). The colloidal iron method is tedious and complicated; picric acid is unsuitable for nitrogen determinations because the reagent itself contains nitrogen; alcohol removes some of the non-protein nitrogen, especially amino acids; and metaphosphoric acid deteriorates on standing. The two precipitants that seem to be best suited for general purposes are trichloroacetic acid and tungstic acid.

Other precipitants have peculiar properties that make them useful for particular purposes. Aluminium hydroxide was found by Marshall and Welker (26) to precipitate all the proteins of blood except oxyhemoglobin. It also removes some creatinine. Alkaline salts of zinc and mercury remove not only proteins, but some non-protein nitrogenous constituents. Because among these nitrogenous compounds are found some of the non-glucose substances which have reducing powers that interfere with the determination of glucose, Somogyi (32) and West, Scharles and Peterson (39) have employed zinc and mercury precipitants in the preparation of blood filtrates for glucose determination. Among the non-protein nitrogenous compounds that are removed by Somogyi's zinc precipitation are uric acid, glutathione, thioneine, and part of the creatinine (33). Urea remains in the filtrate. Benedict and Newton (2) have proposed the use of molybdic acid because it does not precipitate thioneine as tungstic acid does. Except in the determination of thioneine it seems to possess no advantages over Folin and Wu's tungstic acid method, which has proved its reliability in an immense amount of work.

TRICHLOROACETIC ACID PRECIPITATION

Trichloroacetic acid, introduced by Greenwald (16), yields a highly acid filtrate and is the precipitate of choice when such a filtrate is desirable in order to hold in solution for analysis substances, such as Ca and PO_4, which might be removed with the protein coagulum if free acid were not present. The trichloroacetic acid which remains in the filtrate can, when desired, be removed merely by boiling, which splits it into chloroform and CO_2 ($CCl_3COOH = CHCl_3 + CO_2$), volatile products that can be completely driven off by heat. Trichloroacetic acid, compared with tungstic, yields a less voluminous precipitate, and a greater yield of filtrate, which also filters more rapidly (19). Both filtrates yield practically identical results when analyzed for total non-protein nitrogen. High grade commercial trichloroacetic acid is quite satisfactory for most purposes. It can be purified by distillation *in vacuo* at a pressure of 15 to 20 mm.

For the precipitation of blood proteins a 5 or 10 per cent solution of trichloroacetic acid is usually employed. One volume of blood is diluted to 10 volumes with the solution, into which the blood is introduced slowly with constant stirring to secure even precipitation. The mixture is allowed to stand for ten minutes, when the precipitate is removed by filtration or centrifugation. Greenwald (16) has shown that in this concentration trichloroacetic acid removes blood proteins completely without removing any of the compounds usually included under the term non-protein nitrogen.

TUNGSTIC ACID PRECIPITATION BY THE METHOD OF FOLIN AND WU

Tungstic acid, introduced by Folin and Wu (13), yields a nearly neutral filtrate (pH about 6), practically all the acid used being absorbed by the protein precipitate. Folin and Wu have devised methods by means of which the same tungstic acid filtrate can be employed for the determination of all the non-protein nitrogen constituents usually needed and sugar. Other authors have developed methods for the determination of still other constituents in the same filtrates; so that tungstic acid precipitation has become the most generally useful method of eliminating protein. The approximate neutrality of the filtrates and their freedom from heavy metals adapt them even to analyses which involve the action of enzymes; e.g., the determination of urea and fermentable carbohydrate.

The reagents required for the Folin-Wu precipitation are 10 per cent solutions of sodium tungstate and 2/3 or 1/12 N sulfuric acid.

Sodium tungstate reagent. Commercial sodium tungstate ($Na_2WO_4 \cdot 2H_2O$)

varies somewhat in its reaction, but does not contain sufficient impurities to interfere with its use for most purposes. It can be freed from chlorides by recrystallization from alcohol.

If the 10 per cent solution of sodium tungstate is either too acid or too alkaline proteins will not be completely precipitated; therefore the solution must be titrated. If it is alkaline, not more than 0.4 cc. of 0.1 N hydrochloric acid should be required to render 10 cc. of the 10 per cent tungstate solution neutral to phenolphthalein. If it is more alkaline than this, enough acid is added to neutralize it to phenolphthalein. If the tungstate is acid, 10 cc. of the 10 per cent solution is titrated with 0.1 N sodium hydroxide against phenolphthalein until a permanent pink color is obtained. A similar proportion of sodium hydroxide is then added to the remainder of the 10 per cent solution (10).

A 1/12 N sulfuric acid solution. In the original description of the method Folin and Wu (13) advised adding to the blood 7 volumes of water, 1 of tungstate, and 1 of 2/3 N sulfuric acid. Subsequently Folin (11) called attention to the fact that if the acid were not added very slowly a certain amount of the non-protein nitrogen, especially some of the uric acid, was carried down by the precipitate. To avoid this with certainty it proved to be necessary to add the acid in two fractions about twenty to thirty minutes apart. Haden (17) showed that this difficulty could be obviated by combining the acid and water in a single solution of 1/12 N sulfuric acid. This change also simplifies the procedure. Values for non-protein nitrogen obtained by the latest Folin technique and by Haden's modification check accurately.

Haden's modification of the Folin-Wu precipitation

To 1 volume of blood add slowly, with stirring, 8 volumes of 1/12 N sulfuric acid. Then add slowly, with stirring, 1 volume of 10 per cent sodium tungstate solution. Mix well and, after a few minutes, remove the precipitate by filtration or centrifugation.

Van Slyke and Hawkins' (37) modification of the Folin-Wu precipitation

In this procedure the sodium tungstate, sulfuric acid, and water which, in the original Folin-Wu procedure, were added to the blood in succession, are all mixed in advance, so that only one solution is required. The procedure is thereby simplified. Also, for some reason, a larger yield of filtrate is obtained than when the reagents are added separately.

The disadvantage of combining the acid and tungstate is that the mixture is unstable. A precipitate slowly forms in it, and the reagent finally be-

comes too weak to remove blood proteins completely. However, the combined reagent maintains its efficiency for at least two weeks and, where many analyses are made, the convenience of having only one solution to handle more than offsets the disadvantage of having to mix it once a fortnight. When only occasional analyses are made the Haden modification described above is to be preferred.

Reagent

One volume of 10 per cent sodium tungstate is mixed with 8 volumes of 1/12 N sulfuric acid. The solution must be prepared fresh every 2 weeks. The container should be labelled with the date of preparation.

Procedure

To 1 volume of blood add slowly, with shaking, 9 volumes of the combined reagent. The reagent may be added from a burette, or the blood may be placed in a volumetric flask graduated to contain 10 times the volume of the blood sample, and diluted to the mark with the reagent.

TUNGSTIC ACID PRECIPITATION OF UNLAKED BLOOD BY THE METHOD OF FOLIN (12)

Cells contain non-glucose reducing substances which diminish the accuracy of blood sugar methods, and also substances which affect the reagents used for uric acid. To obtain blood filtrates free from such interfering substances, without preliminary centrifugation of the cells, Folin (12) has devised a precipitation procedure in which sodium sulfate is added to prevent laking of the cells. The latter with their non-diffusible constituents, are carried down with the tungstic acid protein precipitate. The method in comparison with the usual Folin-Wu technique yields a larger volume of filtrate, which seems to be free from the cellular constituents that interfere with uric acid determinations, contains little undetermined nitrogen, and less non-fermentable reducing substances than the tungstic acid filtrate obtained from laked blood.

The chief demonstrated value of the method seems to be for uric acid and blood sugar determinations. The method has appeared so recently that there has not been time to ascertain by general use whether it is advantageous for routine analysis.

Reagents

A solution containing 15 grams of anhydrous sodium sulfate, or 38 grams of Glauber's salt, $Na_2SO_4 \cdot 10\ H_2O$, and 6 grams of *sodium tungstate* per liter.

A 1/3 N solution of sulfuric acid.

Procedure

To 8 volumes of sulfate-tungstate solution in a flask, add 1 volume of blood. Mix without rough shaking, which might damage cells, and allow the mixture to stand, with occasional gentle shaking for 5 minutes or longer. Add rather slowly, with constant gentle mixing, 1 volume of 1/3 N sulfuric acid. Centrifuge the mixture at moderate speed for ten minutes. The supernatant liquid which is used for analysis should be colorless and clear.

Although the cells in the precipitate at first are only shrunken, but not disintegrated, on standing they gradually break down. Therefore, the fluid must be quickly removed. Centrifugation is more satisfactory and safer for this purpose than filtration.

BENEDICT'S PRECIPITATION OF PROTEINS WITH TUNGSTOMOLYBDIC ACID (1A)

This procedure completely resembles the tungstic acid precipitation of Folin and Wu, except that Benedict substitutes for the sodium tungstate Folin-Wu solution a mixture of tungstate and molybdate. Benedict has found that whereas tungstic acid precipitates with the proteins part of the ergothionine of whole blood, the tungstomolybdic precipitation leaves this compound entirely in the filtrate. Consequently the tungstomolybdic precipitation should yield a slightly higher non-protein nitrogen content in the filtrate. The difference will hardly be significant for most purposes, however, and it remains to be seen whether the use of the tungstomolybdate reagent in place of the simpler tungstate reagent of Folin and Wu will be justified, except in analyses in which ergothionine is of importance. The precipitation with tungstomolybdate will be described here because it is recommended by Benedict for use with blood sugar and blood uric acid methods described in later chapters. There appears, nevertheless, to be no reason why the Folin-Wu filtrate is not equally applicable to these analyses.

Reagents

Tungstomolybdate solution. Ten grams of pure, ammonia-free molybdic acid are treated in a flask with 50 cc. of N sodium hydroxide solution and the mixture is boiled gently for four or five minutes. The mixture is then filtered, and the filter is washed with about 150 cc. of hot water. The total filtrate is cooled and mixed with a solution of 80 grams of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) dissolved in 600 cc. of water. The mixture of the two solutions is diluted to 1 liter.

0.62 N sulfuric acid. Made by diluting 620 cc. of N sulfuric acid to 1 liter.

Procedure

Whole blood is diluted with 7 volumes of water, and 1 volume of tungstomolybdate solution is added, followed by 1 cc. of 0.62 N sulfuric acid.

Plasma is diluted with 8 volumes of water, and 0.5 volume of tungstomolybdate solution is added followed by 0.5 volume of 0.62 N sulfuric acid.

Blood cells are diluted with 5 volumes of water, and 2 volumes each of tungstomolybdate solution and 0.62 N sulfuric acid are added.

SOMOGYI'S ZINC PRECIPITATION OF PROTEINS WITH REMOVAL OF NON-GLUCOSE REDUCING SUBSTANCES

Somogyi (32) has shown that under certain conditions alkaline zinc salts precipitate from blood not only proteins, but also some of the non-fermentable substances that reduce copper or ferricyanide solutions and therefore interfere with the accurate determination of glucose. The Somogyi zinc precipitation method is described in the chapter on determination of blood sugar. Filtrates secured by this technique from whole blood contain about 10 mg. per 100 cc. less non-protein nitrogen than tungstic acid or trichloroacetic acid filtrates, the difference being chiefly due to the absence of glutathione, thioneine, uric acid, and part of the creatinine (33).

ASHING

Mineral analyses of stools, and sometimes of blood or urine, must be preceded by destruction of the organic matter. The ignition must be sufficiently complete to remove all interfering organic material, but must not be carried out at such a high temperature that the phosphoric acid is converted into meta or pyrophosphate, or that the NaCl and KCl are volatilized. Two ashing procedures have proven their general utility, the wet method of Neumann (27a) and the dry method of Stolte (35).

Neumann's method of ashing with sulfuric and nitric acids *(27a)*. The ashing is carried out in a Pyrex Kjeldahl flask, of 500 to 700 cc. content for macro analyses and 50 or 100 cc. for micro ones. The method can be used either on dry material or on wet.

The weighed or pipetted sample of material is placed in the Kjeldahl flask. For macro analyses 1 to 2 grams of dry feces may be taken, or 5 or 10 cc. of blood. 5 to 10 cc. of a mixture of equal volumes of concentrated nitric and sulfuric acids are added, and the flask is heated gently with a burner. As soon as the evolution of brown fumes diminishes more of the acid mixture is added from a dropping funnel,

and the heating is continued. When it appears probable that the ashing is complete, addition of the H_2SO_4-HNO_3 mixture is stopped, and the heating is continued for a short time until the evolution of brown fumes stops. If the colorless or light yellow solution does not darken or give off gas when the heating is continued the ashing is complete. Then the mixture is cooled and a volume of water, equal to 3 times the volume of acid mixture used, is added. The solution is boiled 5 or 10 minutes to drive off the nitric oxide formed by decomposition of the nitrosyl sulfuric acid.

Urine may be ashed by running it in portions into 1/10 its volume of the HNO_3–H_2SO_4 mixture and boiling down after each addition.

Stolte's dry ashing method, as applied by Tisdall and Kramer (*35, 36*). ***Feces.*** Two grams of the dried and pulverized stools (see p. 78) are weighed into a platinum crucible. This is placed in a quartz or porcelain dish of somewhat larger diameter (usually a dish 10 cm. in diameter and 6 cm. deep is of convenient size), on the bottom of which are placed several pieces of porcelain. The outer dish is gradually heated with a Meeker burner until no more fumes are given off. The purpose of the outer dish is to prevent loss of alkali chloride or change of orthophosphate by overheating the crucible. The flame is finally turned on at full heat, and heating is continued until the charred material is immobile. Then the outside dish is covered, and the heating is continued for one and a half hours. Particles of carbon may remain, but they do not interfere with subsequent analyses. The ash is dissolved in 10 cc. of hot 0.5 N hydrochloric acid and filtered into a 100 cc. volumetric flask. The platinum crucible is washed repeatedly with water, which is passed through the filter into the flask. The volume of solution is finally made up to 100 cc.

Urine. A measured quantity of urine (50 or 100 cc.) is evaporated in a platinum dish, ashed, and extracted as described above for stools. The extract is then made up to the original volume of the urine.

Stolte ashing provides material which may be analyzed for sodium, potassium, calcium, magnesium, and total phosphorus. Some chloride is likely to be lost.

In place of the Stolte arrangement of crucible in a dish, one may heat the crucible in an electric furnace below red heat, at about 400°C. (Shohl, personal communication).

CHOICE OF WHOLE BLOOD, PLASMA, OR SERUM FOR ANALYSIS

No blood constituents are present in the same concentration per liter in cells and plasma. Urea and glucose, however, to both of which human cells

TABLE 11

BLOOD CONSTITUENTS; DISTRIBUTION BETWEEN CELLS AND PLASMA; MATERIAL USED FOR ANALYSIS

BLOOD CONSTITUENT	DISTRIBUTION	CHOICE OF WHOLE BLOOD, SERUM, OR PLASMA FOR ANALYSIS
Urea	Concentration per unit of water same in cells and plasma	Either blood, serum, or plasma
Glucose	Same as urea in human blood	Either blood, serum, or plasma. Cells contain more non-glucose reducing substances than plasma
Creatinine	Apparently same as urea	Either blood, serum, or plasma
Uric acid	Apparently same as urea	Plasma or serum for direct methods because cells contain other substances that react with uric acid color reagents. With silver treatment these are mostly eliminated, so that blood may be used with less error
Amino acids	More in cells	Either blood, serum, or plasma; usually blood
Creatine	Almost entirely in cells	Blood
Undetermined non-protein N	Chiefly in cells	Never determined by itself
Total base*	More per unit volume in plasma. More per gram H_2O in cells	Usually in serum
Sodium*	Almost entirely in plasma of human blood	Serum
Potassium	Chiefly in cells	Usually in serum
Calcium	Chiefly or entirely in plasma	Serum
Magnesium	?	Serum
Bicarbonate and total CO_2†	Concentration per unit volume about 1.7 times as great in plasma as in cells	For acid-base studies preferably serum or plasma; whole blood permissable alternative
Chloride*	Concentration per unit volume about 2 times as great in plasma as in cells	Plasma or serum

* Appreciable deviations from values *in vivo* occur if blood is exposed to air before analysis. Anaerobic handling is desirable.

† Gross deviations from values *in vivo* occur if blood is exposed to air before analysis. Anaerobic handling is required.

TABLE 11—*Concluded*

BLOOD CONSTITUENT	DISTRIBUTION	CHOICE OF WHOLE BLOOD, SERUM, OR PLASMA FOR ANALYSIS
Phosphorus: Total	Chiefly in cells	?
Inorganic	?	Usually in plasma
Acid soluble organic	Chiefly in cells	Blood
Phospholipoids	More in cells	?
Lactic acid	?	Blood, serum, or plasma
Hemoglobin	Entirely in cells	Blood
Oxygen†	Almost entirely in cells	Blood
Plasma proteins	Entirely in plasma	Plasma
Lipoids	Distribution has not been thoroughly studied	Blood, serum, or plasma

appear freely permeable, have been found distributed in direct proportion to the water contents in cells and plasma (see urea and carbohydrate chapters in Volume I), and so far as analytical methods of somewhat uncertain absolute accuracy indicate, the same is true of creatinine and uric acid. The cells contain about 80 per cent as much water per unit volume as the plasma, and accordingly they also contain only about 80 per cent per unit volume as much urea and other freely diffusible non-electrolytes as does plasma. Of such substances the whole blood will contain about 92 per cent as much as plasma per unit volume. This ratio is fairly constant, and for the purposes of most sugar, urea, creatinine, and uric acid determinations one may use either whole blood or plasma or serum. In human blood, in fact, the non-fermentable reducing substances in the cells are sufficient to make the total reducing substances determined in the cells by current blood sugar methods average as high as in plasma. Likewise the cells contain substances other than uric acid which react like the latter with Folin's uric acid reagents, and may make the cells appear to contain even more uric acid than the plasma. In brief, while concentration values for the above four substances can be determined with greater accuracy in the serum and plasma, yet whole blood values are significant for most purposes of these determinations.

In contrast to the above, most of the other blood constituents that have been studied have been found so unevenly distributed between cells and plasma that analysis of whole blood is valueless to indicate the content of such constituents in either cells or plasma. Variations in the proportion of cells in the blood may have more influence on the whole blood analysis for such a substance than changes in the concentration of the latter in either

cells or plasma. Constituents of which this is true are the hemoglobin, plasma proteins, and all the electrolyte ions. Where concentration changes are sought, as in most studies of the electrolytes and acid-base balance, plasma or serum is analyzed for such substances. But where the object is *to find the amount carried in the circulation, as in the case of hemoglobin*, the analysis may be made on the whole blood, regardless of the distribution between cells and plasma.

Table 11 indicates types of determinations in which more valuable information can be secured by examining plasma or cells rather than whole blood.

DETERMINATION BY HEMATOCRIT OF THE VOLUME OF CELLS IN BLOOD

An hematocrit is essentially a graduated centrifuge tube. Hamburger (18) proposed the use of a conical centrifuge tube, to the lower end of which was sealed a graduated tube of narrow bore. A measured small amount of blood, diluted with a known volume of some isotonic solution, is placed in the tube and centrifuged at high speed until the cells, which are entirely in the narrow graduated portion of the tube, have been compressed to a minimal volume. This method has been followed by numerous observers.

Others have recommended centrifuging blood in fine graduated capillary tubing. This obviates dilution and is no less exact. The tubes must be supported in the centrifuge in such a manner that they are whirled in a horizontal position and in line with the center of motion of the apparatus. Therefore, if they are to be swung in the ordinary centrifuge cups they must be supported in some manner. The special Daland hematocrit attachment adapted to International Instrument Company centrifuges is quite satisfactory.

The tubes should be of such fine bore and so short that they can be filled by capillarity. The bore should be uniform. To test this a small amount of mercury is introduced and measured at different positions in the tube. The ends of the tube should be carefully ground, not fused.

The total length of the tube is first measured. By measuring the length of the cell column after centrifuging, the per cent cell volume may be estimated. The tubes as purchased are graduated into 100 parts. In the authors' experience the graduation is usually quite regular, but the total length of the tubes is often less than 100 units, probably because they are ground after they have been graduated. The regularity of the calibration and the length of the tubes must, therefore, be determined by measurement with a fine scale or accurate calipers. This can best be done by measuring the distances from 90 to the zero end, from 10 to 100 end and from 10 to 90. The actual value of calibrations within the practical range can then be calculated.

Procedure

If the exact volume of cells per 100 cc. of circulating blood is desired, blood must be collected with anaerobic precautions and prevented from coagulating by one of the procedures which does not alter cell size (see section on anticoagulants, above). The addition of potassium oxalate to make a 0.3 per cent solution causes the cells to shrink by about 2 per cent of their volume (7).

The blood is introduced as rapidly as possible into the tube so that it is completely filled. The ends of the tube are then sealed by a heavy rubber band. The tube is placed in the centrifuge and whirled at a speed of 2000 to 3000 r.p.m. until the cell volume has reached a minimum value. Usually forty-five to sixty minutes are required. The cell mass should, at this point be absolutely translucent, indicating that the cells are so closely packed that there is no refraction from the interstices. Before the volume is read care must be taken to ascertain that none of the blood has been lost from the tube during centrifugation.

If all these precautions are observed and the tubes are properly graduated, reproducible values for cell volume may be obtained with an error not usually exceeding 1 volume per cent.

Cell volume can also be determined by mixing blood with isotonic (1.6 per cent) sodium oxalate (see Hooper, Smith, Belt, and Whipple in chapter XXII) or sodium chloride solution (0.9 per cent, see Shock and Hastings in chapter XXVII) and centrifuging in a tube with a calibrated capillary at the bottom to measure the cells.

DIRECT AND INDIRECT DETERMINATIONS OF CELL CONSTITUENTS

If it is desired to ascertain the concentration of any constituent in blood cells two methods may be employed.

1. The direct method, in which cells and plasma are separated by centrifugation and analyzed separately.

2. The indirect method, in which whole blood and plasma are analyzed separately and cell volume is determined by means of the hematocrit. From the cell volume and the difference between the analytical results yielded by whole blood and plasma, the concentration in the cells may be calculated. Because in this method the error in calculating any cell constituent is the sum of errors in both cell and plasma analyses, to which is added also the error in hematocrit determination, the indirect method is capable of less accuracy for cell constituents than the direct.

Direct method by separate analysis of cells and plasma. The blood is centrifuged at high speed until the volume of cells no longer changes. This usually requires from thirty to forty-five minutes. For analysis as much plasma is removed from the packed cells as can be obtained without stirring up the latter. The last remaining drops of plasma and the upper layers of cells are then removed separately and discarded. Samples of the packed cells are then taken for analysis.

Although it appears that thorough centrifugation can drive out all but a slight proportion of serum from the interstices between the cells, the removal can not be absolutely quantitative. Consequently even cell data secured by direct analysis can not be considered entirely exact. It is probable, however, that the effect of the adherent serum is within the limits of error of most analysis made on cells.

Indirect method by separate analysis of whole blood and plasma and cell volume determination. Plasma is separated from a portion of the blood by centrifugation. Both whole blood and plasma are separately analyzed. The cell volume is then determined by hematocrit. The concentrations in cells and plasma are then calculated by the following formulae in which C_b, C_c and C_p represent concentration in blood, cells, and plasma respectively, and V = volume of cells expressed as a fraction of total blood volume:

1. C_p and C_b are directly determined by analysis.

2. $$C_c = \frac{C_b - [C_p'(1 - V)]}{V}$$

An idea of the effect on C_c of errors in the three determinations from which C_c is calculated in the indirect method, may be gained from the following example. If C_b is 50 volumes per cent of CO_2, C_p is 60 volumes per cent of CO_2, and V is 0.43, all ordinary normal values, one calculates by Equation 2 that C_c, the CO_2 content of the cells, is 36.8 volumes per cent. An error of + 1 volume per cent in C_b will increase the calculated C_c by 2.3 volumes per cent; an error of − 1 volume per cent in C_p will increase C_c 1.4 volume per cent; an error of + 0.01 in V will increase C_c by 0.5 volume per cent. If all three errors should occur simultaneously they would raise the calculated C_c to 40.9 volumes per cent, or 111 per cent of its correct value. If the difference between C_b and C_p is less a given error in either causes a greater error in the calculated C_c. Likewise as V departs in either direction from the value 0.50 an error of 0.01 in it causes an increasingly greater error in the calculated C_c.

PRESERVATION OF URINE

Within a short-time, urine, unless it is collected and preserved with precautions against contamination, undergoes changes because of the

action of bacteria upon the urine solutes. Chief among these changes are the production of ammonium carbonate from urea and other nitrogenous compounds, and the decomposition of sugar. If sufficient ammonium carbonate is produced to turn the urine alkaline, some ammonia may be volatilized.

Urine voided with alkaline reaction has a considerable bicarbonate content, sufficiently important among the total urine buffers to make the $BHCO_3:H_2CO_3$ buffer influential in determining the urine reaction (see acid-base chapter in "Interpretations" volume). When such urine is exposed to air it loses CO_2 and becomes still more alkaline.

Methods employed for the preservation must depend on the purposes for which the urine is intended. Collection of complete specimens, even by catheter, without any bacterial contamination can not be accomplished with certainty. Subsequent additional contamination may be minimized by using only urinals and receptacles which have been carefully scrubbed and cleaned by sulfuric bichromate cleaning mixture or sterilized by boiling or in an autoclave. Decomposition is retarded by cold; therefore specimens not analyzed at once should be placed in the refrigerator as soon as possible after collection. Determinations of unstable constituents should be begun as soon as possible.

To minimize loss of CO_2 urine must be collected with the least possible exposure to air, by the method outlined for "Determination of Urine Bicarbonate," in Chapter VII.

If analysis for total nitrogen is intended, it is necessary merely to keep the reaction strongly acid in order to fix the ammonia. This can be accomplished by acidifying the fresh urine with a small amount of strong acid, either sulfuric or hydrochloric.

This method of preservation is not, however, suitable if analysis for acid excretion or ammonia is intended. In this case the use of urinals scrupulously cleaned by the methods outlined above, rapid transfer to similarly cleaned stoppered receptacles which are immediately placed in the refrigerator, and analysis at the earliest possible moment must be relied upon. A suitable preservative may further retard decomposition.

The preservatives most commonly employed for routine purposes are toluol, thymol, and chloroform. Of toluol enough is added to make a thin film over the surface of the urine. A crystal of thymol large enough to saturate the urine with the drug distinctly retards the growth of microorganisms. A small amount of chloroform has a similar effect, but interferes with certain analyses.

Short (31) recommends chinosol as extremely satisfactory in a concentra-

tion of $\frac{2}{3}$ gram per liter of urine. He also finds a mixture of 2 parts by weight of hexamethylenamine and 1 part of acetyl-salicylic acid effective in a concentration of 5 grams per liter.

COLLECTION, PRESERVATION AND TREATMENT OF FECES IN PREPARATION FOR CHEMICAL EXAMINATION

Marking stools

Feces formed during a definite time interval can be marked by feeding some material which will color them. For this purpose carmine and charcoal have been used.

A 5-grain capsule of carmine is given with the first meal of a metabolism period and again with the first meal after the period has ended. All stools after the first dose of carmine are collected and marked with the date and hour of passage. All are inspected for the red color of carmine and are discarded until this appears. After this time all stools are preserved, including the one that first contained carmine, until the appearance of the second carmine. The period ends with the last stool before the appearance of carmine from the second dose. Periods of two or more days are commonly used.

Perservation of stools. It is preferable to have the stools sent in the original receptacles to the laboratory, where they are at once placed in the refrigerator. As soon as possible they should be transferred from the pans to containers in which they may be collected and stored. Two-quart Pyrex glass bean pots with lids make excellent containers. Urine which may have been voided in the pans is poured off and may be treated like other urine. Toilet paper, if not greatly contaminated, may be removed. The solid or semi-solid matter is then transferred to the bean pot as completely as possible with the aid of a hard rubber sink scraper and the smallest possible amount of water. If nitrogen determinations are desired, a suitable quantity of strong sulfuric acid is first poured into the pot. The pot, covered, is kept in the refrigerator or under a hood.

Because fecal material is solid or semi-solid and heterogeneous it must be subjected to some preparation that will make it possible to secure a representative sample. For this purpose the stool may either be dried or pulverized, or it may be suspended in sulfuric acid. The latter method is more rapid and convenient, but is accompanied by carbonization of organic matter, so that it can not be used in preparing stools for estimation of fat or other organic substances. On the other

hand, the sulfuric acid suspension previous to nitrogen determination is preferable to drying, because during drying ammonia is likely to be lost.

Drying and mixing. The feces in a weighed bean pot or evaporating dish, are dried over a steam bath under a hood. The mass should be stirred from time to time. When it is almost dry a small amount of alcohol is stirred in and the drying process repeated. In order to get a mass that can be pulverized it may be necessary to repeat the addition of alcohol and redrying. The dried feces and the container are weighed. The mass is then pulverized and passed through a sieve. The dried and thoroughly mixed powder may be placed in a stoppered bottle for subsequent analysis.

Preparation of stool suspension in sulfuric acid. For determinations of nitrogen and the mineral constituents other than chloride and sulfur, the prolonged and repugnant process of drying and pulverizing stools can be avoided by suspending the fresh material in strong sulfuric acid. In this a charred suspension or solution is formed which is sufficiently homogeneous to be sampled with pipettes.

The twenty-four hour stool is placed in a 2 liter Pyrex beaker or bean pot, and about 300 cc. of water are added. The mixture is stirred rapidly, with a mechanical stirrer, while 250 cc. of concentrated sulfuric acid are slowly added. The mixture becomes hot, and the organic matter is dissolved and charred so that a homogeneous black suspension results. Water is cautiously added to make the volume about 900 cc. The mixture is cooled, transferred to a 1 liter flask and made up to volume, a few drops of caprylic alcohol being added to prevent foaming.[1]

Following is an alternative procedure. It does not yield a mixture suitable for pipetting, but can be carried through without a mechanical stirrer, and without volatilizing chloride.

The feces are transferred from the original receptacles, at the earliest possible moment, to Pyrex bean pots containing enough concen-

[1] The above technique was devised by MacKay and Butler in a study, not yet published, of mineral metabolism in nephritis in the Hospital of the Rockefeller Institute.

It was also used successfully in determining the nitrogen and mineral contents of *foods*. The three meals of the day's diet are mixed with 1000 to 1500 cc. of water and 1000 cc. of concentrated sulfuric acid is slowly run in with rapid mechanical stirring, which is continued for a half-hour after the last portion of sulfuric acid has been added.

trated sulfuric acid to render the contents strongly acid and to digest partially the solid matter. The acid prevents escape of ammonia. At the end of the experimental period the material is diluted with enough water to make a fairly liquid mixture. If the pots are not large enough for all the stools the latter are transferred to a larger vessel with the aid of the water. The material is mixed thoroughly and then passed through a coarse wire sieve. The material retained by the sieve is washed and ground in the sieve. The minute residue which remains is ground up in a mortar with more acid and water. The total homogeneous, acidified watery suspension thus obtained is weighed. Portions may be weighed out, after the suspension has been well shaken, and used for analyses with which the presence of sulfuric acid does not interfere.

For the Kjeldahl determination of total nitrogen it is unnecessary to add as much sulfuric acid as usual because of the amount which has already been introduced in the above procedures.

PREVENTION OF FOAMING

Because of the protein and certain other organic matter which they contain, biological solutions of all kinds have a tendency, when they are agitated, to form foam at the surface which may interfere with accurate volumetric measurement or lead to loss of fluid. To prevent foaming various neutral substances which reduce surface tension have been used.

To remove foam from the surface of urine or blood in order to read a meniscus a drop or two of ether may be added just before the reading or before the solution is diluted to the mark. Or a wire wet with caprylic alcohol may be touched to the foamy fluid surface in a burette, flask, or pipette.

If foaming must be prevented during continued agitation of the fluid, —e.g., in the aeration process of urea determination or the evacuation of blood in gasometric analyses—caprylic alcohol appears to be the most satisfactory reagent. One or two drops usually suffice. Satisfactory preparations of ***caprylic alcohol*** can be secured on the market or can be prepared in the following manner:

Castor oil, mixed with an equal volume of concentrated sodium hydroxide is allowed to stand over night. The mixture is then distilled in an oil bath, the temperature of which is gradually raised to 250°. A liter of castor oil yields about 200 cc. of caprylic alcohol. *The oil used for the bath must have a flash point well above 250°.*

Amyl alcohol has been used as a substitute for caprylic, but is less satisfactory.

In certain circumstances—e.g., aeration in urea determinations—after agitation of a solution has continued for a long period, foaming may commence in spite of the presence of caprylic alcohol, and the addition of a further amount of the anti-foam reagent may be ineffectual. In this case the introduction of a few drops of ethyl alcohol is usually effective.

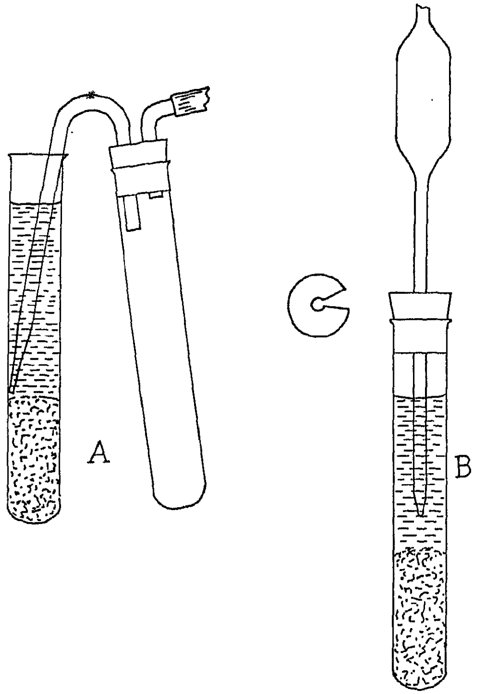


FIG. 8. Device to remove fluid from precipitates. *A*, as much supernatant fluid as possible is desired for analysis. *B*, a measured volume of the fluid is desired. The tip of the tube in the fluid may be drawn to a capillary and bent back in a U to prevent disturbing the precipitate when fluid is withdrawn.

Fiske (9) finds that isoamyl isovalerate or a mixture of equal parts of this substance with isoamyl alcohol are especially useful to prevent foaming. Mitchell and Eckstein (27) recommend phenyl ether.

REMOVAL OF SUPERNATANT FLUIDS FROM PRECIPITATES AFTER CENTRIFUGATION

It is frequently necessary, as in the separation of serum or plasma, to remove supernatant fluid from centrifuged precipitates without decantation.

It is desirable in such cases to make the separation as completely and cleanly as possible without risk of disturbing the precipitate. For this purpose several expedients are employed.

One of the best of these, involving the use of mercury sampling bulbs has already been described on page 55. It is adapted particularly to the anaerobic removal and preservation of serum and plasma.

For the removal of serum from centrifuged cells a pipette with a long rubber tube interposed between it and the mouth may be used. This permits the technician to observe the position of the tip of the pipette throughout the procedure. By compression of the tubing he can check the flow when he desires. Provided heavy walled tubing is used, the method serves for rough purposes, but does not permit the most complete separation because of the tendency for small amounts of fluid to run back when the tubing is compressed.

A better procedure is illustrated in figure 8, *A*. It utilizes a tube which is large enough to hold all the supernatant fluid and is equipped with a two-holed rubber stopper. Through one hole passes a fine-bore, heavy-walled bent glass tube with its free end drawn to a slender capillary. Through the other hole passes a shorter glass tube with a rubber suction tube attached. With the capillary point in the supernatant fluid, suction is exerted. Under visual control the point may be moved around as desired to collect the fluid without disturbing the precipitate. When the fluid has been removed as completely as possible the tip is withdrawn without interrupting suction, so that there is no back flow and all the fluid in the glass capillary is drawn into the collecting tube. If a small amount of precipitate is inadvertently drawn into the capillary, suction may be discontinued, whereupon the fluid remaining in the capillary returns to the original tube, washing the precipitate back with it.

By a device similar to that illustrated in figure 64, chapter IX, less complete removal of the supernatant fluid can be effected without risk of disturbing the precipitate. The capillary tube with tip curved upwards is inserted into the liquid as far as is possible without disturbing the precipitate. The fluid can then be blown or sucked out through the capillary without contamination by the precipitate.

To remove a measured portion of supernatant fluid a pipette fitted firmly into a cork or rubber stopper may be used. With the stopper resting on or in the top of the tube, the tip of the pipette is inserted through the stopper to the proper distance beneath the fluid (see figure 8, *B*). If the

stopper is fitted into the neck of the container the stopper should have a perforation to admit air as the fluid is withdrawn.

BIBLIOGRAPHY

1. Austin, J. H.; Cullen, G. E.; Hastings, A. B.; McLean, F. C.; Peters, J. P., and Van Slyke, D. D.: Studies of gas and electrolyte equilibria in blood. I. Technique for collection and analysis of blood, and for its saturation with gas mixtures of known composition. *J. Biol. Chem.*, 1922, **54**, 121.

1*a*. Benedict, S. R.: The analysis of whole blood. I. The precipitation of proteins. *J. Biol. Chem.*, 1931, **92**, 135.

2. Benedict, S. R., and Newton, E. B.: The use of molybdic acid as a precipitant for blood proteins. *J. Biol. Chem.*, 1929, **82**, 5.

3. Dautrebande, L.; Davies, H. W., and Meakins, J.: The influence of circulatory changes on the gaseous exchanges of the blood. III. An experimental study of circulatory stasis. *Heart*, 1923, **10**, 133.

4. Denis, W., and Beven, J. L.: Methods of preservation of specimens of blood intended for the determination of the nonprotein organic constituents. *J. Lab. Clin. Med.*, 1924, **9**, 674.

5. Drucker, P., and Cullen, G. E.: A simple method for obtaining cutaneous (capillary) blood from infants and adults for colorimetric pH determinations. *J. Biol. Chem.*, 1925, **64**, 221.

6. Eisenman, A. J.: The effect of potassium oxalate on electrolytes of blood and plasma. *J. Biol. Chem.*, 1927, **71**, 587.

7. Eisenman, A. J.: Method for anaerobic defibrination of blood. *J. Biol. Chem.*, 1927, **71**, 607.

8. Evans, C. L.: Acid production in shed blood. *J. Physiol.*, 1922, **56**, 146.

9. Fiske, C. H.: The inhibition of foaming. *J. Biol. Chem.*, 1918, **35**, 411.

10. Folin, O.: Note on the necessity of checking up the quality of sodium tungstate used in the system of blood analysis. *J. Biol. Chem.*, 1922, **51**, 419.

11. Folin, O.: A system of blood analysis. Supplement IV. A revision of the method for determining uric acid. *J. Biol. Chem.*, 1922, **54**, 153.

12. Folin, O.: Unlaked blood as a basis for blood analysis. *J. Biol. Chem.*, 1930, **86**, 173.

13. Folin, O., and Wu, H.: A system of blood analysis. *J. Biol. Chem.*, 1919, **38**, 81.

14. Foster, G. L.: Studies on carbohydrate metabolism. I. Some comparisons of blood sugar concentrations in venous blood and in finger blood. *J. Biol. Chem.*, 1923, **55**, 291.

15. Goldschmidt, S., and Light, A. B.: A method of obtaining from veins blood similar to arterial blood in gaseous content. *J. Biol. Chem.*, 1925, **64**, 53.

16. Greenwald, I.: The estimation of non-protein nitrogen in blood. *J. Biol. Chem.*, 1915, **21**, 61; *Ibid.*, 1918, **34**, 97.

17. Haden, R. L.: A modification of the Folin-Wu method for making protein-free blood filtrates. *J. Biol. Chem.*, 1923, **56**, 469.

18. Hamburger, H. J.: Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes. Berlin, 1912.

19. Hiller, A., and Van Slyke, D. D.: A study of certain protein precipitants. *J. Biol. Chem.*, 1922, **53**, 253.

20. Howell, W. H.: The purification of heparin and its chemical and physiological reactions. *Johns Hopk. Hosp. Bull.*, 1928, **42**, 199.

HOWELL, W. H., AND HOLT, E.: Two new factors in blood coagulation, heparin and prothrombin. *Am. J. Physiol.*, 1918, **47**, 328.

21. HÜRTER: Untersuchungen am arteriellen menschlichen Blute. *Deut. Arch. klin. Med.*, 1912, **108**, 1.
22. JOHN, H. J.: Preservation and transportation of blood for chemical study. *Arch. Path.*, 1926, **1**, 227.
23. KUBIE, L. S.: The solubility of O_2, CO_2, and N_2 in mineral oil and the transfer of carbon dioxide from oil to air. *J. Biol. Chem.*, 1927, **72**, 545.
24. LAX, H., AND SZIRMAI, I.: Ein Verfahren zur Haltbarmachung des Blutes für die Blutzuckerbestimmung. *Münch. med. Woch.*, 1929, **76**, 58.
25. MAJOR, R. H.: Potassium fluoride as a preservative for blood. *J. Am. Med. Ass.*, 1923, **81**, 1952.
26. MARSHALL, J., AND WELKER, W. H.: The precipitation of colloids by means of aluminium hydroxide. *J. Am. Chem., Soc.*, 1913, **35**, 820.
27. MITCHELL, H. H., AND ECKSTEIN, H. C.: A foam inhibitor in the Van Slyke amino nitrogen method. *J. Biol. Chem.*, 1918, **35**, 373.

27*a*. NEUMANN, A.: Eine Veraschungsmethode (Säuregemischveraschung) und vereinfachte Bestimmung von Eisen, Phosphorsäure, Salzsäure, und anderen Aschenbestandteilen unter Benutzung dieser Säuregemischveraschung. *Z. physiol. Chem.*, 1902–1903, **37**, 115.

28. OSTERBERG, A. E., AND SCHMIDT, E. V.: The influence of sodium fluoride and thymol on the determination of urea by the urease method. *J. Biol. Chem.*, 1928, **76**, 749.
29. ROE, J. H.; IRISH, O. J., AND BOYD, J. I.: The preservation of blood for chemical analysis by the use of sodium fluoride. *J. Biol. Chem.*, 1927, **75**, 685.
30. SANDER, F. V.: The preservation of blood for chemical analysis. *J. Biol. Chem.*, 1923, **58**, 1.
31. SHORT, J. J., AND PIATETSKY, A.: Note on urine preservatives. *J. Lab. Clin. Med.*, 1926, **12**, 1098.
32. SOMOGYI, M.: A method for the preservation of blood filtrates for the determination of sugar. *J. Biol. Chem.*, 1930, **86**, 655.
33. SOMOGYI, M.: Nitrogenous substances in zinc filtrates of human blood. *J. Biol. Chem.*, 1930, **87**, 339.
34. STADIE, W. C.: The oxygen of the arterial and venous blood in pneumonia and its relation to cyanosis. *J. Exp. Med.*, 1919, **30**, 215.
35. STOLTE, K.: Eine einfache und zuverlässige Methodik der Aschenanalyse. *Biochem. Z.*, 1911, **35**, 104.
36. TISDALL, F. F., AND KRAMER, B.: Methods for the direct quantitative determination of sodium, potassium, calcium and magnesium in urine and stools. *J. Biol. Chem.*, 1921, **48**, 1.
37. VAN SLYKE, D. D., AND HAWKINS, J. A.: A gasometric method for determination of reducing sugars, and its application to analysis of blood and urine. *J. Biol. Chem.*, 1928, **79**, 739.
38. VAN SLYKE, D. D.; WU, H., AND MCLEAN, F. C.: Studies of gas and electrolyte equilibria in the blood. V. Factors controlling the electrolyte and water distribution in the blood. *J. Biol. Chem.*, 1923, **56**, 765.
39. WEST, E. S.; SCHARLES, F. H., AND PETERSON, V. L.: The determination of true sugar in blood. *J. Biol. Chem.*, 1929, **82**, 137.

CHAPTER III

ANALYSES OF GAS MIXTURES

DISCUSSION

Interpretation of gas analyses

The *composition of a gas mixture* is ordinarily expressed in terms of the *volume of each gas present in 100 volumes of the dry mixture.* This volume is termed the *volume per cent*, or simply the *per cent*, of each gas.

When a gas mixture is analyzed in the ordinary way, by successive removal of the constituent gases and estimation of each from the shrinkage in gas volume, the per cent of any gas calculated directly from the observed volume represents the proportion of that gas in the total mixture of *dry* gases, even when the analysis is performed, as usual, on a gas mixture saturated with water vapor. In the analysis of moist air by the Haldane method, for example, each volume measurement, before or after absorption of any gas, is made at the same partial pressure of the dry gases. This pressure is, $B - W$ (B = barometric pressure, W = vapor tension of water). The effect of the vapor tension, W, is therefore merely to lower the pressure for all volume readings by a constant amount, and thereby to increase all volume readings by a constant *proportion* over what they would be for the dry gases. When a certain amount of gas is removed, as when O_2 is absorbed, a proportional amount of water vapor condenses with it. Consequently when gases are determined moist each makes up the same proportion of the total mixture as when they are measured dry. The results of a gas analysis are therefore independent of temperature, barometric pressure, and wet or dry state of the gas, so long as all of these factors are constant.

Gas samples and sampling

The process of sampling consists of drawing a gas sample from a spirometer or other source into a sample container. The container must be so formed that transfer of gas to it from the original container, and from it to the gas analysis apparatus, can be performed conveniently and without admixture of significant amounts of atmospheric air.

The sample container must have for connection, first with the source of gas and later with the gas analysis apparatus, a capillary outlet tube provided with a three-way cock. This cock makes it possible, after the con-

tainer is connected with the gas analysis apparatus, to displace the air in the connecting capillary with either mercury from the apparatus or gas from the container. The container must also be provided with a leveling bulb or other device by means of which gas withdrawn in successive samples can be replaced by mercury, water, or other fluid. Gas containers which answer these requirements are shown in figures 9; 10, I, 1; 10, II; and 11.

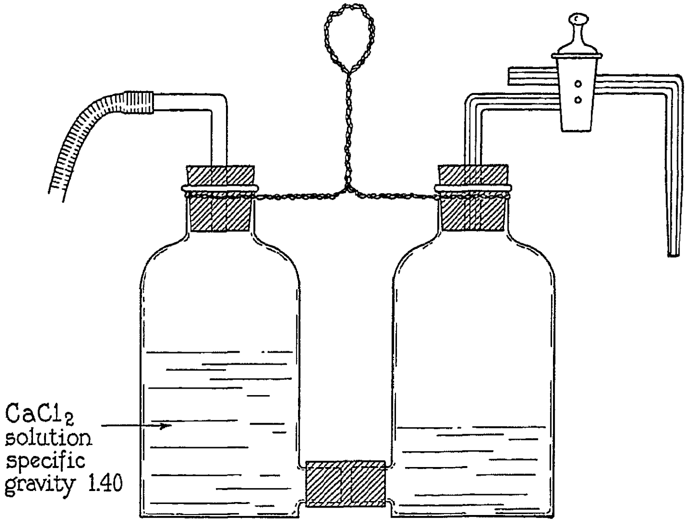


FIG. 9. Temporary container for gas samples. The aspirator bottles are of 250 or 500 cc. capacity. The liquid used is calcium chloride solution of 1.40 specific gravity in which CO_2 is only $\frac{1}{16}$ as soluble as in water. Expired air can be kept over the solution in this container for three or four hours without change in CO_2 content, but not for longer unless the container has previously contained expired air so that the calcium chloride solution is partially saturated with it. Mixtures of O_2, N_2, and CO without CO_2 can be kept for several days.

It is essential that the fluid used to displace withdrawn gas be one in which none of the gases in the mixture to be analyzed shall be soluble. Mercury is the perfect fluid in this respect. Its weight and cost, however, make it desirable to use water or salt solutions when possible. Water can usually be used as fluid in contact with gas mixtures containing O_2, N_2, and CO, which are not very soluble, if the contact is not prolonged more than a working day. Carbon dioxide, however, is so soluble in water that contact of respired air with water can not be permitted for even a brief time without significant absorption of CO_2. If, however, the water is nearly saturated with calcium chloride, and slightly acidified, the solubility coefficient of this

gas at room temperature is lowered from about 1.0 to 0.06.[1] In the container shown in figure 9 respired air in contact with such a solution, even freshly prepared and free of CO_2, does not change in CO_2 content enough to affect an ordinary Haldane analysis within three or four hours. In a longer time, however, measurable absorption of CO_2 by the calcium chloride solution occurs, unless the solution has previously been allowed to stand in contact with respired air (unpublished notes of the authors). The container in figure 9 has proved useful for holding samples of respired air from Tissot spirometers in work where the analyses were made within the same day the samples were obtained.

In general, mercury is the displacement fluid to be relied upon for all gases and for indefinite periods of time, and one is justified in using other fluids only when one has satisfied oneself that they introduce no inaccuracies under the particular conditions employed.

Before sampling, the sample container, including as a rule the connecting capillary, is filled with fluid (usually mercury), and is then connected with the source of gas. The care with which atmospheric air must be excluded from the connections depends somewhat upon the amount of gas available. If there are many liters, as in the case of gas from a Tissot spirometer, a fraction of a cubic centimeter of air in the tubes connecting container and sampler is of no consequence. Gas drawn into the sampling tube can be passed back and forth to the container, so that the effect of a little air in the connecting tube is diluted into insignificance. Or some gas from the container can be wasted through the three-way cock of the sampler, to wash air out of the connections. However, when only a small excess of gas is present, as may occur in collection of Haldane alveolar air samples, such dilution or washing out of the connecting tubes may not be practicable. In this case the connecting tube attached to the sampler, and also the tap of the vessel from which the gas is taken, must be filled with mercury, to the complete exclusion of air.

Gas Analyses with the Haldane Apparatus

THE APPARATUS

The Haldane gas analysis apparatus in its original form (6) or modifications (3, 8, 11, 14) thereof is now in practically universal use for exact air

[1] Carbon dioxide has at room temperature the approximate solubility coefficients in different fluids: water, 0.90; ammonium sulfate solution, 0.22; saturated NaCl solution, 0.24; saturated $CaCl_2$ of 1.44 specific gravity, 0.07; $CaCl_2$ solution of 1.40 specific gravity, 0.08; glycerol, 0.31. Most organic solvents other than glycerol dissolve more CO_2 than does water. Calcium chloride solution of 1.40 specific gravity consists of approximately 40 grams $CaCl_2$ + 60 grams H_2O, or 80 grams $CaCl_2 \cdot 6\,H_2O$ plus 20 grams H_2O.

analysis. Modifications especially precise are those of Carpenter (3, 4) and Krogh (11), in which air can be analyzed for O_2 and CO_2 with errors less than 0.01 volume per cent. Such apparatus requires skillful handling in order to yield the precision of which it is capable. In most laboratories one finds either the Haldane model (fig. 10, I) which takes 10 cc. samples and yields results accurate to about 0.03 volume per cent, or modifications which differ only in details of convenience.

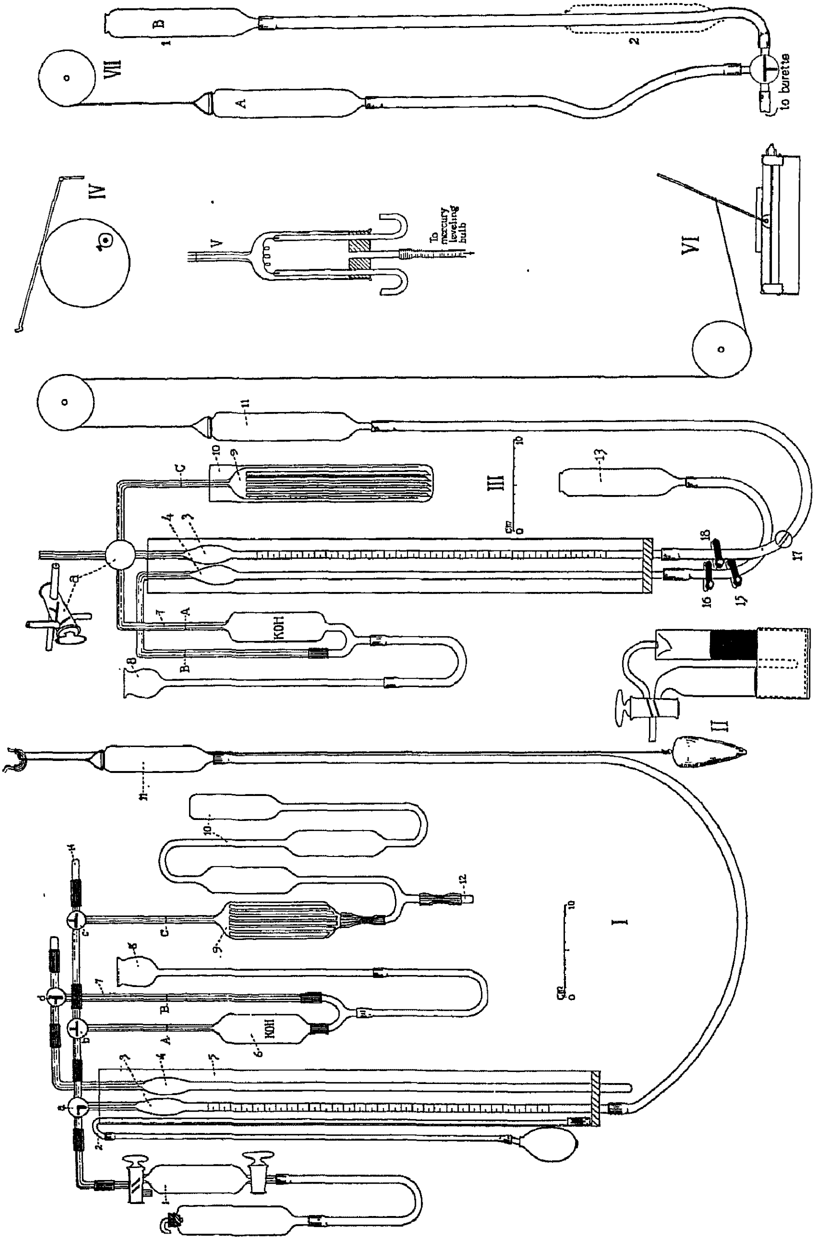
Figure 10, I, represents schematically the Haldane gas analysis apparatus equipped for the determination of oxygen and carbon dioxide. Figure 10, III, represents a modification of this apparatus which has only one cock, of the four-way type designed by Y. Henderson (8), and has jointless all-glass connections between the gas burette and the absorbing tubes for O_2 and CO_2. This modification offers an important advantage in that the possibility of leaks is reduced to a single place, the one cock. It is the form used by the authors.

In Newcomer's (14) modification the entire apparatus, absorption tubes as well as gas burette, is enclosed in one large flat water bath, so that all parts are at one temperature. This is theoretically an advantage, but whether it actually yields more exact results than the types shown in figure 10 appears to be not yet shown.

The apparatus in all its modifications consists essentially of a gas burette, *3*, fitted with a stop-cock, *a*, through which the gas may be admitted to the burette and passed into the absorbing chambers, of which there are two. One of these, *6*, contains potassium hydroxide to absorb CO_2; the other, *9*, contains pyrogallol to absorb O_2. The burette is enclosed in a water-jacket, *5*, which also contains a stirrer, *2*, and a thermobarometer, *4*.

The *stirrer* consists of a small glass-tube with its lower end closed by a rubber cap in which a slit acts as a Bunsen valve. By means of an atomizer bulb or connection with a compressed air system air is bubbled through the water rapidly enough to stir it efficiently, without splashing.

The *thermobarometer* consists of an uncalibrated tube similar in size and shape to the gas burette and connected by a side arm, *7*, with the potassium hydroxide reservoir. This device eliminates the necessity of correcting for changes in temperature or pressure during an analysis. Before each reading of the burette the levels of fluid in the burette connection, *A*, and in the thermobarometer connection, *B*, are adjusted to the same marks, so that the volume of gas in the thermobarometer remains always the same, regardless of changes in temperature and barometric pressure which may occur during an analysis. The gas in the burette is thereby put under pressure which maintains its volume likewise constant, except for changes due to removal of CO_2 and O_2.


I
II
III
IV
V
VI
VII
KOH
KOH
To burette
To mercury leveling bulb
cm

The capillaries between the burette and absorbers are made of thick-walled glass tubing of 1.5 to 2.0 mm. bore. This is sufficiently wide to allow free passage of gas or fluid, and not wide enough to create an undesirably great dead space unprotected by the water jacket.

The *carbon dioxide absorber* consists of a simple reservoir bulb, *6*, filled with KOH solution, connected by the side arm, *7*, with the thermobarometer and, by an outlet below, with a long-stemmed thistle tube, *8*, through which fluid can be introduced or withdrawn, and by which the level of fluid in the reservoir may be controlled. The stem of the thistle tube is usually supported by a spring clip that allows it to be set at any desired level.

Because pyrogallol absorbs oxygen slowly the *pyrogallol reservoir*, *9*, is so constructed as to expose a large surface of fluid to contact with the gas. This is effected in the usual Haldane apparatus by the introduction of a number of fine glass tubes. There is a tendency, when the pyrogallol becomes old and thick or develops a precipitate, for these tubes to become obstructed so that bubbles of air become imprisoned in them. When this occurs the absorber must be cleaned out and fresh pyrogallol introduced. In order to protect the pyrogallol from contact with air, strong alkali, which may be covered with a layer of mineral oil, is kept in the tubes marked *10* in diagram I, of the original Haldane. In the modified apparatus, figure 10, III, the pyrogallol is protected from air only by a layer of paraffin oil directly on the pyrogallol in tube *10*.

A convenient innovation, also illustrated in figure 10, III, is the introduction of a leveling bulb attached to the lower end of the thermobarometer tube. The lower end of the thermobarometer here contains mercury covered by a short column of water. The latter insures saturation of the contents of the tube with water vapor. Mercury is allowed to enter the tube to a level somewhat above the rubber connection, effectually sealing the latter. The rubber connecting tube is kept closed during a determination by means

---

FIG. 10. Diagrammatic representation of the Haldane air analysis apparatus and various modifications. For general description see text.

I. Original Haldane apparatus assembled for use (43).

II. Bailey gas sampling tube (3).

III. An adaptation of Y. Henderson's (49) modification of the Haldane apparatus.

IV. Automatic mixing device to be run by motor.

V. Combustion chamber for use with Haldane apparatus in determination of hydrogen and other combustible gases. For description see "Determination of hydrogen" on page 100.

VI. Automatic wind-shield wiper used as mixing device.

VII. Arrangement of leveling bulbs and stop-cock to permit rapid and accurate control of mercury level in burette of Haldane apparatus.

of a screw clamp or stop-cock, *15*. The thermobarometer may be set before a determination by opening the screw clamp, *15*, and adjusting the height of the leveling bulb, *13*. Screw clamp *16* is always partially open to be used in fine adjustment, as discussed below.

Mechanical devices of all kinds have been suggested to replace tedious raising and lowering by hand of the leveling bulb during the absorption of oxygen. Of these one is illustrated in figure 10, VI. An automatic wind shield cleaner, activated by air suction or compression, can be used to serve two Haldane machines at the same time. The length of thrust can be regulated by adjusting the attachment of the string at the proper distance from the center of rotation of the movable arm of the cleaner. On a large scale, a battery of Haldane apparatuses can be served by a single motor running a shaft on which are attached at proper intervals eccentric wheels like that illustrated in figure 10, IV, each supporting a hinged rod to the end of which a leveling bulb may be attached. Whatever mechanical device is employed must transmit an even motion to the leveling bulb with not more than twenty, and preferably fewer, oscillations per minute.

For *finer adjustment of the level of mercury* in the burette the leveling bulb of the original Haldane apparatus was hung from a rack and pinion device. Satisfactory control can be secured, however, by merely attaching a counterweight to a cord which supports the leveling bulb and passes over a hook which supplies enough frictional resistance to prevent the string from slipping so long as the counterweight approximately balances the bulb. Such a device is illustrated in figure 10, I.

Although not indispensible, cock *17* and clamp *18* (fig. 10, III) are of decided assistance in the finer adjustment. By means of the leveling bulb, *11*, the mercury in the burette is brought to such a level that the KOH or pyrogallol is at approximately the level *A* or *C*. Glass cock *17* is then closed, and screw clamp *18*, which is routinely left partly open, is screwed together or apart until the adjustment is exact. Clamp *16* is similarly used on the thermobarometer to bring the KOH to the level *B*.

Another convenient method of adjustment is shown in figure 10, VII. Two leveling bulbs are connected with the burette by means of a three-way stop-cock. One of these, *A*, is left permanently attached to the automatic mixing device. For the other, *B*, two hooks are provided: *1*, high enough to drive mercury into either the burette or the other bulb, *A*; *2*, low enough so that mercury will run into it from either the burette or bulb *A*. When it is desired to drive the gas into and from an absorber, bulb *B* is set in position *1* and the desired quantity of mercury is admitted from it to the burette. The stop-cock is then turned to connect the burette with bulb *A* and the

automatic mixer is started. When the residual gas is to be measured the stop-cock is turned to allow mercury to run into *B*, which has been set in position *2*. From time to time it is necessary to adjust the volume of mercury in *A* by exchange between *A* and *B*. The one disadvantage of this system is that the mercury tends to become more rapidly soiled by grease from the stop-cock.

For *lubrication of stop-cocks* vaseline or a mixture of vaseline and lanoline is quite satisfactory except in hot weather, when a combination of rubber stop-cock grease (see p. 47) and vaseline is more satisfactory. The stop-cocks should be lubricated with a minimum of grease, none of which should be allowed to lodge in the bores. They must turn without resistance and should not be subjected to pressure when they are used (see lubrication of stop-cocks, p. 47).

All *rubber tubing used for connections* should be of pure gum, and with sufficiently heavy walls to prevent kinks. It must be sulfur-free, especially the tubing that comes in contact with the alkali, which might acquire oxygen absorbing power if it dissolved sulfur. The tubing in contact with the alkali solution is cleaned and then soaked three or four times, for an hour each time, in alkali of the same concentration with which it is to be used.

Calibration of the Haldane burette

The burette of the Haldane apparatus has a volume of approximately 10 cc., of which the bulb and the uncalibrated portion above the bulb hold a little less than 7 cc., while the lower, calibrated portion holds the remaining 3 cc. This lower portion is graduated in scale divisions of 0.01 cc., running completely around the tube and sufficiently far part to permit reading by interpolation to 0.001 cc. The exact volume of the burette is of no importance; it is important that each scale division of the graduated portion equal 0.001 of the total volume. The burette must be calibrated so accurately that the corrections are determined with an error not exceeding ± 0.0001 of the volume, or 0.001 cc. To insure such accuracy it is necessary to calibrate the burette with mercury instead of water. The burette is used wet, and a film of moisture on the walls should therefore be present when the apparatus is calibrated.[2] The meniscus read is the top of the mercury; there may be a little visible water about the edge of the mercury meniscus, but not more.

The burette of the *original type of Haldane apparatus* (fig. 10, I) *is calibrated with inclusion of the volume occupied by the bore of the stop-cock,* since the air trapped in the bore is passed with the air from the rest of the burette

[2] See section on calibration with mercury in the preceding chapter.

into the KOH and pyrogallol tubes in the analysis. In the Henderson type of apparatus (fig. 10, III), on the contrary that part of the gas sample which is trapped in the intake bore of the four-way cock remains there throughout the analysis. Consequently *in calibrating a burette with the Henderson cock the bore of the cock is not included.*

The burette when purchased is usually provided, for use in calibration, with a calibrating stop-cock, sealed on to the lower end.[3] Below the cock is a capillary tip drawn to a fine, smooth point to deliver drops of mercury not exceeding 0.005 cc. (0.07 gram) in size.

The burette is scrupulously cleaned with warm bichromate-sulfuric acid cleaning mixture, and rinsed with distilled water.[4] The burette is set up for calibration in clamps in a rigid, vertical position, with care to avoid bending strain. The cock at the top is turned to connect the interior of the burette with the outer air. By suction from the top a little water is drawn into the calibrating tip below. The latter is then immersed in clean mercury, which with the aid of a pump is drawn by suction *slowly* up into the burette, under control by the lower stop-cock. As the mercury rises a layer of water collects on its surface. Suction is continued until this water and a little mercury have been drawn over into the suction tube. If the mercury has been drawn up slowly, the water film adherent to the walls will be just the right amount. The greater part of the mercury is now permitted to flow out from the lower cock, and is again slowly drawn up into the burette, and a little into the suction tube, as before. By these two fillings with mercury all the water is displaced from the burette, except the slight film which remains permanently.

When burette, upper stop-cock, and glass capillary above the cock, are completely filled with mercury, the calibrating cock at the bottom of the burette is closed, and the suction tube above is disconnected. Mercury is run out from the calibrating cock until the meniscus in the upper capillary has sunk to the top of the stop-cock, in the original Haldane apparatus (fig. 10, I). If the burette has a Henderson cock (fig. 10, III), it is turned to admit air from one of the side tubes before the delivery of mercury for weighing is begun.

[3] It is not satisfactory to use a stop-cock attached to the burette by rubber tubing because the tubing expands as the height of the mercury in the burette increases. Even binding the tubing with tape or wire does not entirely eliminate this source of error.

[4] Alcohol and ether may be used as grease solvents in the burette at this time. Because of their volatile nature they must not be introduced into the apparatus after it has been assembled for use. Alcohol and ether vapors are eliminated from the assembled apparatus with difficulty and seriously affect gas analysis.

Mercury is now delivered into a weighing bottle until the meniscus falls to the 7 cc. mark. A reading to 0.001 cc. is made and the mercury weighed. Further readings and weighings are made at 0.1 cc. intervals until the 10 cc. mark is reached. The results are checked by repeating the process.

A correction curve is then prepared. In the preparation of this curve it is not necessary to calculate absolute volumes; one may more simply use proportions of the total volume. For example, if the mercury held in the burette to the 10 cc. mark is 135.7 grams and that to the 7 cc. mark is 94.5, then the capacity at the 7 cc. mark is $\frac{94.5}{135.7} \times 10 = 6.964$ tenths of the total capacity. The correction to be applied is, therefore, $6.964 - 7.000 = -0.036$ cc. In this case the unit used as a "cc." is one-tenth the total capacity of the burette, and is exactly the standard cubic centimeter only if the burette at the 10 cc. mark contains exactly 10 cc.

Assembling the apparatus

Original Haldane Type. The original Haldane apparatus illustrated in figure 10, I, is usually sold in a portable case. For laboratory use, however, most workers prefer a permanent set up, which can be improvised by any ingenious technician to suit his convenience. All glass parts are carefully aligned and fixed in position without strain, but sufficiently rigid to prevent motion in joints. To minimize dead space, motion, and leaks, all connections are made "glass to glass," with the least possible space between the opposed ends of glass tubing. The rubber connecting tubes should not be bound with wire. Wire is not as effective for this purpose as rubber bands. No binding at all is necessary if a fine grade of tubing without traces of bloom is used. If the peripheries of the glass tubes near the ends are covered with a small amount of rubber-containing stop-cock grease (p. 47) before the rubber connecting tube is applied, the latter will become sealed to the glass so fast that the likelihood of a leak will be minimol.

Before the apparatus is assembled, about 2 cc. of water are drawn up into the thermobarometer tube (fig. 10, I, *4*).

After all glass parts, scrupulously cleaned, have been set in position, stop-cocks ***b***, ***c*** and ***d*** (fig. 10, I) are removed and the pyrogallol and hydroxide are introduced into their respective reservoirs. The potassium hydroxide for CO_2 absorption is slowly run in through the thistle-tube, *8*, until the reservoir, *6*, is half full. All air bubbles are expelled from the connecting tubes by alternately raising and lowering the thistle tube.

When this has been accomplished more alkali is slowly added until, with the thistle tube elevated, the fluid level stands in about the middle of the stem of the thistle tube and rises in the capillary tubes to the level of the etched marks, *A* and *B*. Stop-cocks *b* and *d* are now replaced in position.

Potassium hydroxide is run into 10 (fig. 10, I) until the bulb nearer the oxygen absorber is two-thirds full, when a small amount of mineral oil is added. Finally pyrogallol is introduced through *12*, very slowly until it reaches the level of the etched mark, *C*. Care must be taken that no bubbles of air are imprisoned in any of the small glass tubes in the absorber. Stop-cock *c* is now placed in position.

Stop-cock *a* is removed, and it and the adjoining connections are cleaned, then *a* is replaced. The leveling bulb, *11*, and its rubber tubing are attached to the burette and bound on with rubber bands (no grease should be used in making these connections). Clean mercury is introduced by way of the leveling bulb, which is alternately raised and lowered to drive all air from the tubing. The mercury is permitted finally to rise in the burette to the opening in stop-cock *a*, when the leveling bulb should be about one-third full. A few drops of water slightly acidulated with sulfuric acid are drawn into the burette through stop-cock *a*. The mercury in the burette is lowered so that the water moistens the walls; then the mercury is slowly raised and the excess water is ejected through the upper cock, on to a piece of filter paper. There should, as during calibration, be just enough water left in the burette to make a visible ring about the mercury surface when the latter is near the 10 cc. mark.

Henderson type. The setting up of the Henderson modification of the Haldane apparatus in figure 10, II, is similar, but is simpler, because there is only one glass cock and no rubber connections between the gas burette and the KOH and pyrogallol tubes.

The filling of the thermobarometer is quite different in the Henderson type, where the thermobarometer has a leveling bulb of its own (fig. 10, III, *13*). Water is placed in this leveling bulb, and about 3 cc. are run into the thermobarometer (fig. 10, III, *4*). The KOH bulb is then filled. Then water is withdrawn from the thermobarometer into its leveling bulb until the KOH solution in the connecting capillary rises to the mark *B*. Thereupon the connection with the leveling bulb is closed with the clamp on the rubber tube.

Setting the apparatus

Elimination of oxygen and carbon dioxide from the apparatus is a necessary preliminary to its use. It is effected by drawing air into the gas burette, and running it back and forth alternately to the KOH and pyrogallol tubes, as in analysis of gas mixtures, until all the O_2 and CO_2 in the burette and connecting tubes have been absorbed, and only nitrogen remains in the apparatus.

After the gas in the apparatus has been rid of oxygen and carbon dioxide, the pyrogallol is adjusted to the mark *C*, figure 10, I, or 10, III, by altering the level of mercury in the burette by means of leveling bulb *11*, with stop-cocks so set that the burette communicates with the pyrogallol, but is excluded from the hydroxide. The burette is then connected with the bulb of potassium hydroxide, *6*. By alternately altering the levels of leveling bulb *11* and thistle-tube *8* the hydroxide is brought to the marks *A* and *B*. At this point the hydroxide should stand at about the middle of the stem of the thistle tube. This gives maximum flexibility in the adjustment of the thermobarometer against temperature changes. Alkali is put into or removed from the thistle tube by means of a pipette with drawn out tip until the proper amount of fluid is present. If the thermobarometer is equipped with a leveling bulb, as in figure 10, III, this is used to bring the alkali to a convenient level. The burette is finally reconnected with the oxygen absorber. If the pyrogallol meniscus moves much from *C* the process of setting is repeated until the pressures in the potassium hydroxide and the pyrogallol are as nearly as possible the same, so that, on connecting the burette alternately with hydroxide and pyrogallol, the fluid levels shift least from the marks *A*, *B* and *C*.

The same end can be attained in the original Haldane apparatus by making a first setting with stop-cock *b* open to burette, hydroxide, and pyrogallol, and bringing the fluid levels as nearly as possible to the three marks *A*, *B* and *C* at the same time by adjusting leveling bulb *11* and thistle tube *8* (or the thermobarometer leveling bulb); but this can not be done with the Henderson cock.

ANALYSIS OF RESPIRATORY AIR FOR CO_2, O_2, AND N_2

Reagents

Potassium hydroxide, 10 per cent, for the absorption of carbon dioxide. The solution should be entirely clear. If a precipitate is present the solution should be filtered through asbestos or discarded, because the particles might block the capillaries of the apparatus.

Stirring. The fluid in the bath is thoroughly stirred by bubbling air through it. This is a necessary preliminary to each setting or reading of the apparatus. Stirring must not be so vigorous that fluid is splashed on to the tubing above the water. Such fluid would cool the tubing by evaporation.

Setting the apparatus and measuring the sample. The burette is now connected with the hydroxide reservoir and the apparatus is rapidly set as before, so that the hydroxide levels are at the marks *A* and *B* when burette and hydroxide are in communication. (The pyrogallol level should not have deviated from *C* during the introduction of the sample.) After a burette reading has been taken the water bath is again stirred, and the reading is repeated, with the burette and hydroxide bulb still connected and the hydroxide levels at *A* and *B*. This is again repeated if necessary to obtain a constant reading, but reasonable speed is necessary to complete the setting before CO_2 diffuses into the hydroxide. If such diffusion begins successive settings will yield steadily decreasing readings, and the analysis must be abandoned.

Absorption of CO_2. The gas burette is left connected with the KOH tube, and by raising and lowering the leveling bulb the gas is driven about 5 times to and from the hydroxide solution. The apparatus is then reset, the water bath is stirred, and the burette reading is recorded.

In order to make sure that all CO_2 has been absorbed the gas is driven twice more into the hydroxide and another reading is made. This process is repeated until two successive readings do not differ.

Or the gas may be driven back and forth mechanically for whatever length of time is found to ensure complete absorption of CO_2.

Each time the top of the falling mercury column reaches the narrower lower portion of the bulb and enters the contracted graduated tube its rate of descent becomes greatly accelerated. If care is not exercised at this point the mercury may fall so rapidly that it overshoots the mark, drawing alkali into the connecting capillary. In this case the analysis must be discarded and the apparatus must be cleaned in the manner described below before it can be used again.

Absorption of oxygen. After CO_2 absorption is finished the burette is connected with the pyrogallol tube, and the gas is driven repeatedly into the latter as it was into the hydroxide. However, absorption of O_2 proceeds much more slowly than absorption of CO_2. If the gas is driven back and forth by hand, it is worth while to let it remain several seconds in the pyrogallol tube each time before it is returned to the burette. At least 10 exchanges are necessary to remove even the major portion

of the oxygen. It is in the oxygen absorption that automatic devices for raising and lowering the mercury bulb are most welcome. If such a device is used the mercury is allowed to rise into the bulb of the burette before the leveling bulb is attached to the automatic elevator. The latter must be so regulated that the motions of the mercury meniscus in the burette are confined entirely to the wider upper portion of the burette; otherwise either mercury or pyrogallol may be driven into stop-cocks and connecting tubes. When most of the oxygen has been absorbed the mixer is stopped and the leveling bulb lowered until the pyrogallol rises approximately to mark *C*. The number of passages required by hand, or the number of minutes with a mechanical mixer, in order to attain complete absorption of oxygen is ascertained by trial. Even more care must be taken in lowering the leveling bulb after absorption of oxygen than after the absorption of carbon dioxide because of the smaller amount of gas which now remains. The residual gas is now passed two or three times into the hydroxide to rinse out oxygen remaining in the connecting tube *A*. The hydroxide is brought back approximately to the mark *A* and the gas is again driven repeatedly into the pyrogallol as before. The pyrogallol is now brought exactly to the *C* mark, the apparatus is then set in the usual manner with burette and hydroxide bulb connected and a reading taken. After this the gas is again passed several times into the pyrogallol. The apparatus is set and another reading taken. This process is repeated until two successive readings agree.

Calculation

$$\frac{100\ (R_T - R_{CO_2})}{R_T} = \text{per cent of } CO_2$$

$$\frac{100\ (R_{CO_2} - R_{O_2})}{R_T} = \text{per cent of } O_2$$

$$\frac{100\ R_{O_2}}{R_T} = \text{per cent of } N_2$$

R_T = initial reading of burette, made when it contains the total gas sample.
R_{CO_2} = reading after CO_2 has been absorbed.
R_{O_2} = reading after O_2 has been absorbed.
In analyses of respired air duplicate CO_2 determinations should differ by

not more than 0.03 per cent, O_2 determinations by not more than 0.04 per cent.

DETERMINATION OF HYDROGEN

Of the clinical procedures described in this volume, only the lung volume method of Van Slyke and Binger (p. 226) requires determination of hydrogen. In this method a mixture containing H_2 and N_2 in more or less nearly equal proportions is analyzed. The determination of hydrogen will accordingly be described for application to such a mixture.

The pyrogallol tube in the Haldane apparatus is replaced by a tube of similar size, figure 10, V, but closed at the bottom by a paraffined cork, securely wired on. If the tube is to be used with the original type of Haldane apparatus (fig. 10, I), a three-way cock is sealed on at the top, as in the case of the pyrogallol tube *9* of figure 10, I. With the Henderson type of Haldane apparatus tube *9*, III, of figure 10 can be converted into a combustion tube by removing the pyrogallol and fine tubes, and corking it at the bottom. The cork is pierced by 3 glass tubes. One connects with a mercury leveling bulb. The other two carry heavy sealed-in platinum wires, connected within the bulb by a spiral of about three turns of thin (26 or 28 gauge) platinum wire. A current of about 4 volts, obtained from two or three dry cells, is used to heat this wire to redness during the analysis. To economize in platinum the tubes into which the wires are sealed can be curved at the bottom, as shown in figure 10, V, and can be filled with mercury before the cork is inserted; or, instead of mercury, the tubes can be filled with molten Wood's metal into which copper wires are sealed. This device, introduced by Dr. J. Sendroy, is the most convenient (see fig. 13, p. 131). The mercury or Wood's metal serves to make a connection between the battery wires and the platinum ones, so that the latter need only be long enough to reach through the walls of the tubes.

If hydrogen forms more than 9 per cent of a gas mixture containing also enough oxygen to combine with the hydrogen, the mixture is explosive. Consequently it is essential, in order to secure a quiet combustion, to have less than 9 per cent of hydrogen present. Mixtures containing more hydrogen are diluted with oxygen or air before analysis.

For the analysis of such hydrogen-nitrogen mixtures as are encountered in the lung volume determination the gas to be analyzed is diluted with 5 to 8 volumes of air. For this purpose about 8 cc. of air and then about 1.5 cc. of the $N_2 - H_2$ mixture are measured into the Haldane burette in the following manner: Air is first admitted, and then the sample container shown in figure 10, I, *1*, is attached to the burette. A small mercury leveling bulb

is attached to the outlet of the three-way cock of the container, and from it the connecting tubes leading to the burette are filled with mercury. In the case of apparatus I the bore of the burette cock itself is not filled with mercury, but in the case of apparatus III the bore of the cock is so filled. The volume of air in the burette is then read after setting the apparatus. Then the desired volume of the hydrogen-containing gas is admitted from the sample container, and measured by the increase in the volume in the burette. If CO_2 is present in the gas it is first removed by passing the gas back and forth into the KOH bulb, and the burette is again set and read.

TABLE 12

Behavior of Gases on Combustion

GAS	CONCENTRATION OF GAS IN AIR REQUIRED TO FORM EXPLOSIVE MIXTURE AT ATMOSPHERIC PRESSURE*	COMBUSTION		
		Reaction	Shrinkage in gas volume on combustion	CO_2 formed
	per cent		*vols. per 1 vol. of the gas*	*vols. per 1 vol. of the gas*
Hydrogen	9.0	$2\ H_2 + O_2 = 2\ H_2O$	1.5	0
Carbon monoxide	16.0	$2\ CO + O_2 = 2\ CO_2$	0.5	1.0
Methane	6.0	$CH_4 + 2\ O_2 = 2\ H_2O + CO_2$	2.0	1.0
Ethylene	4.0	$C_2H_4 + 3\ O_2 = 2\ H_2O + 2\ CO_2$	2.0	2.0
Acetylene	3.0	$2\ C_2H_2 + 5\ O_2 = 2\ H_2O + 4\ CO_2$	1.5	2.0
Benzene	2.5	$2\ C_6H_6 + 15\ O_2 = 6\ H_2O + 12\ CO_2$	2.5	6.0
Water gas	12.0			
Coal gas	8.0			

* According to Haldane (6).

The greater part of the gas is then driven over into the combustion chamber, and the platinum wire is heated. The gas is moved back and forth over this wire a number of times until all the hydrogen is burned. The combustion tube is permitted to cool and the gas is returned to the burette and measured. It is then passed to and from the KOH bulb, to mix with hydrogen left in the capillaries leading to that bulb, and the combustion is repeated. This process is repeated until no further shrinkage in gas volume occurs.

Calculation

Since in the reaction, $2\ H_2 + O_2 = 2\ H_2O$, the shrinkage in gas volume is 3/2 of the hydrogen volume, the shrinkage observed in the analysis is multiplied by 2/3 to give the hydrogen.

$$\text{Volume per cent } H_2 = 100 \times \frac{2/3 \text{ volume decrease by combustion}}{\text{Volume of sample}}.$$

Since the sample constitutes only about 1/6 of the gas, the error of analysis is about ± 0.1 volume per cent, compared with 0.02 to 0.03 in the CO_2 and O_2 determinations in expired air.

GASES OTHER THAN HYDROGEN BY COMBUSTION

A number of gases besides hydrogen can be determined by combustion carried out in connection with either the Haldane or the manometric apparatus. The carbon containing gases can be identified by the relationships between the volume of the gas, the shrinkage on combustion, and the amount of CO_2 formed, which is readily determined in the burned gas. Table 12 shows these relationships, and the minimum proportion of each gas in air which is stated by Haldane (6) to produce a mixture that is explosive at atmospheric pressure.

PRECAUTIONS IN USE OF HALDANE APPARATUS

The chief difficulties encountered in the use of the Haldane apparatus are: 1, keeping the burette clean; 2, preventing mercury or absorbing reagents from entering connections or stop-cocks; 3, imprisonment of bubbles in absorbers; 4, leaks; 5, partial obstruction of connecting tubes and stop-cocks; 6, lack of enough water in the burette.

Keeping the burette clean. If the burette is not scrupulously clean accurate analyses are impossible. No visible particles nor droplets of mercury or water should adhere to its walls when the mercury column is lowered. The chief cause of a soiled burette is dirty mercury, and the factors mainly responsible for contamination of mercury are dust from the air and contact with dirty rubber tubing, with grease, or with amalgamating metals. To keep the burette clean mercury used in the apparatus should be free from amalgam and scrupulously clean before it is introduced (see "cleaning mercury," p. 45). To guard the mercury against access of dust it is well to stopper each leveling bulb with a loose plug of cotton or with a rubber stopper with a curved glass tube (fig. 10, I, *1*). This tube must be large enough to permit unimpeded access of air, and the bloom must be carefully removed from the stopper by scrubbing. Only the highest grade rubber tubing prepared as directed on page 48 should be used for the leveling bulb connection: it should be attached without grease or copper wires, and made fast with the aid of rubber bands. Mercury should never be passed unnecessarily through the gas burette's stop-cock, which should be lubricated with a minimal amount of grease.

Cleaning the burette. Even if all these precautions are observed the burette will still need cleaning from time to time. If its condition is not too bad it can be washed by introducing through the top, by means of a funnel and a short rubber connection, a mixture of equal parts of concentrated nitric acid and water. This is permitted to stand in the burette for thirty minutes. It is then expelled. The burette is rinsed repeatedly with distilled water and finally with water slightly acidulated by the addition of a few drops of sulfuric acid. Of the last all is expelled except the slight amount necessary to wet the walls of the burette. Stop-cock *a* is then removed and it and its connections are thoroughly cleaned with the aid of a pipe cleaner. It is then regreased and reinserted.

If this treatment is not sufficient the leveling bulb must be disconnected and fresh bichromate-sulfuric solution drawn up into the burette by suction exerted through a rubber tube connected to the top. When the burette is filled the rubber tube is closed with a screw clamp. The solution may be allowed to stand in the burette for some hours. (It is safer during this period to remove stop-cocks *b* and *c*, figure 10, I). The cleaning solution is then rinsed out of the burette repeatedly with distilled water and finally with slightly acidulated water. Stop-cock *a* and its connections are cleaned in the usual manner. It may at times be necessary to clean also the rubber tubing by the method prescribed for the preparation of new tubing (see p. 48).

If *mercury is driven through the stop-cock* into the connecting tubes to the absorbers, mercury droplets are likely to lodge in the capillaries and exert a valve-like action. This results in the production of differences of pressure on the two sides of the droplet and makes accurate check readings impossible.

If *alkali or pyrogallol is sucked back* into the burette, or if alkali is drawn even so far as half-way through the connecting capillary towards the burette, an experience which no operator will always escape, the burette and its connections must be washed with a mixture of 1 N sulfuric acid. Stop-cocks through which the alkali has passed must be removed, washed, and regreased.

The washing can usually be effected without renewing the absorbent solutions. In the original Haldane apparatus (figure 10, I) the burette is filled with acid, plug *14*, at the end of the manifold tube is removed, and cocks *b* and *c* are set as in figure 10, I. The manifold tube and stop-cocks are rinsed with 1 N sulfuric acid from the burette. Stop-cocks *b* and *c* are then removed, and they as well as the adjoining parts of tubes *A* and *C* are cleaned with acid and water with the aid of a pipe cleaner. By the same means

CO_2 and 20.93 ± 0.02 volumes per cent of O_2 (3, 4, 6). In New York in winter, when immense amounts of coal are being burned, the CO_2 may rise to 0.10 per cent and the O_2 sink to 20.85–20.90, but in less densely built-up places constancy of composition of the outdoor air may be assumed.

Gas Analyses with the Manometric Apparatus

APPARATUS AND TECHNIQUE OF MEASUREMENTS

The manometric apparatus described in detail in chapter VII was designed by Van Slyke and Neill (25) primarily for determination of gases in blood. The apparatus, however, has proven to be equally convenient for analyses of respiratory air and other gas mixtures, and in the technique of its handling is for some analyses simpler than the Haldane apparatus. In accuracy for analysis of the CO_2 and O_2 in respiratory air it equals the usual 10 cc. form of the Haldane apparatus, the error being in the neighborhood of 0.02 to 0.04 per cent. For air with small amounts of CO_2 or CO the manometric apparatus permits the use of a technique which gives results to nearly 0.0001 per cent of an atmosphere, a sensitiveness not attainable with the Haldane apparatus. It is convenient to have both apparatuses available. The Haldane is slightly more rapid in analyses of respired air. When a manometric apparatus is at hand, however, it is possible with it alone to carry out all the gas analyses required in ordinary physiological or clinical studies.

Whereas in the Haldane apparatus amounts of gas are measured by the volumes which they occupy at barometric pressure, in the manometric apparatus the gas is brought to an arbitrarily chosen convenient volume and the amount is determined by the pressure which it exerts on a manometer. With the usual Haldane apparatus one can not measure amounts of gas filling less than 70 per cent of the burette. With the manometric apparatus amounts can be measured which at atmospheric pressure would cover the volume range from 0.1 cc. to 35 cc. Even the 0.1 cc. portion can be measured with an accuracy of 1 part in 300 while the 35-cc. portion can be measured to nearly 1 in 5000. One can, therefore analyze samples of a greatly varying range of size in a single manometric apparatus.

Apparatus

The manometric apparatus and the general technique for handling it are described on pages 267 to 282 in chapter VII. We shall in this chapter describe only the particular manipulations required for analyses of gas mixtures. If the analyst is not already familiar with the apparatus he should read the above pages. For gas analysis the apparatus, if made before 1931,

may require modification by extending the scale of the manometer downwards a few centimeters, as described below.

Extension of manometer scale for low p_o readings. The zero readings with the mercury in the chamber at the 50 cc. mark fall in the low part of the manometer tube opposite the bottom of the chamber. Most of the manometers made before 1931 have scales which fail by 10 or 20 mm. to extend so low, since none of the methods used prior to this time involved zero readings with more than 2 cc. of gas space in the chamber. It is, however, not difficult to improvise an extension of the scale to make possible zero readings at the lower point. On transparent paper a ladder of parallel lines 1 mm. apart is made with black India ink. Each line is made long enough to extend half way around the manometer tube, except that every fifth line is made a little longer to facilitate counting the distances. The strip of paper with the lines is cut of sufficient width to extend two-thirds of the way around the manometer tube. It is then pasted onto the tube in such a way that the open third is towards the observer's eye, and the uppermost mark on the paper covers the zero mark on the glass scale. If such a scale extension is used, a convenient way to employ it in connection with the scale already present is to add 100 mm. to each reading made on the latter, and consider the zero point to be the point on the extrapolated scale 100 mm. lower than the original zero of the glass scale.

Technique of manometer readings

Before every manometer reading in these gas analyses the mercury meniscus in the chamber is lowered below either the 2 or the 50 cc. mark, and is then brought slowly up to the mark by admitting mercury from the leveling bulb while the latter rests level with the bottom of the chamber. While the mercury in the chamber is rising to the mark it is observed with a magnifying glass (a good reading glass serves well), and the cock from the leveling bulb is closed just as the top of the curved mercury meniscus reaches the mark. With practice one can bring the mercury in the chamber thus to the same level with a constancy Of 0.05 mm. It is essential that the mercury surface should always be brought to the mark in the same manner from below upwards, and never from above downwards, because the manometer readings obtained after these two different approaches differ, slightly but measurably. After the mercury is on the mark in the chamber the reading on the manometer is taken. If there is any doubt concerning the accuracy of the placing of the mercury meniscus in the chamber the mercury is lowered and brought to the mark again, and the reading is repeated.

In such duplicate observations the readings on the manometer (also with the help of a lens) should differ by not more than 0.1 mm.

Temperature control and corrections

Temperature control. The manometric apparatus is used under conditions to minimize temperature changes during the short time required for an analysis. If the temperature registered by the thermometer in the water jacket of the chamber of the apparatus differs from that registered by a thermometer in the air of the room at the same level, the water jacket is warmed or cooled by wrapping it for a minute or longer in a towel wet with hot or cold water. The chamber is then shaken and the temperature on its thermometer noted. It is desirable to bring it within 0.2 or 0.3° of the room temperature. During an analysis the room temperature is kept as constant as possible.

Correction for temperature change during an analysis. A gas analysis is usually performed by measuring the pressure, P_S, of the sample, removing one gas by absorption or combustion, and then measuring the pressure, P_R, of the residual gas. The 2 manometer readings by which P_S is measured are taken so quickly after one another that there is seldom a significant temperature change between them, and likewise the 2 readings by which P_R is determined. During the operations between the P_S and P_R measurements, however, the temperature, read on the thermometer in the water jacket of the manometric chamber, may change by over 0.1°. In such a case the observed P_R is corrected by multiplying it by the factor T_S/T_R, where T_S and T_R represent the absolute temperatures (centigrade + 273°) of the gas chamber at the times of the P_S and P_R measurements, respectively. The P_R thus corrected is the pressure the residual gas would exert at the temperature of the P_S measurement.

Admission of sample estimated by volume

For most gas analyses one takes a sample sufficient to give 450 to 550 mm. pressure at either 2 or 50 cc. volume, according to whether a micro or macro analysis is to be done.[5] Such a sample can be measured with sufficient accuracy as a rule by the following procedure.

The manometric chamber is washed as described on page 236 if

[5] For special purposes a manometric chamber, calibrated for volumes such as 10 or 20 cc., instead of the 0.5 and 2.0 cc. calibrations of the standard Van Slyke-Neill apparatus, may be desirable. For the gas analyses described in this chapter, however, the standard chamber, used also for determination of gases extracted from blood and other solutions, has been found adequate..

necessary to free it from interfering solutions used in preceding analyses. Then all the water present is removed except the invisible film adherent to the walls. To remove excess water the mercury in the chamber is lowered to the bottom, and then is permitted to rise *slowly* to the top. The water which collects on the surface is expelled, and about 2 cc. of

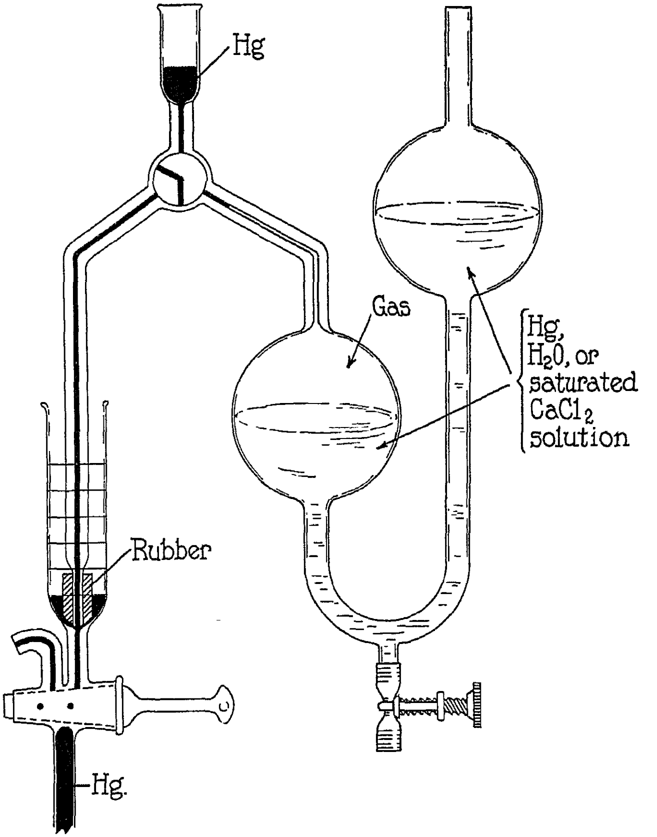


FIG. 11. Admission of gas sample from modified Hempel pipette to chamber of manometric apparatus. The Hempel pipette, provided with three-way stop-cock; serves for storage of gases or of liquids out of contact with air. In the case of gases the upper bulb is filled with fluid, as above. When air-free solutions are to be stored mercury takes the place of water and the solution takes the place of the gas in the illustration. The bulbs are each of about 50 cc. capacity; the capillary has a bore of 1.0 ± 0.1 mm. When the pipette is standing with stored gas or solution a little mercury is admitted into the capillary leading to the lower bulb to seal the 3-way cock and prevent leakage around it. From Van Slyke and Hiller (24).

mercury are driven up into the cup above the chamber. The chamber is then evacuated and the mercury lowered to the 50 cc. mark. If more water then collects on the mercury surface than will form a slight ring about the edge, the above procedure is repeated to finish the removal of the water. The repetition will not be necessary unless during the first removal the mercury was allowed at some point to rise too rapidly to permit the water to detach itself completely from the glass walls ahead of the ascending mercury.

The *zero reading* of the apparatus p_o, with the chamber free of gas and visible water, is now taken, as described above under "Technique of manometer readings," with the mercury meniscus at either the 2 or the 50-cc. mark, whichever is to be used in the analysis.

To *admit the gas sample* the tip of the capillary from the sample container is inserted into the mercury in the cup of the chamber, as shown in figure 11. Either the container there shown may be used, or containers of the type shown in figures 9 or 10, I. The connecting capillary should be narrowed at the tip, which is fitted with a rubber ring as shown in figure 11. Such a ring is made by cutting a section about 10 mm. long from a soft rubber tube of about 1 mm. bore with walls 2 mm. thick. The bottom of the ring is beveled slightly by grinding on an emery wheel or with sandpaper, so that it will fit the curvature of the bottom of the cup when pressed into the latter as shown in figure 11.

The manometric leveling bulb is first placed in the ring above the chamber (see fig. 37; the manometric leveling bulb is not shown in figure 11), and mercury is forced up through the connecting capillary and three-way cock of the sample container, displacing the air in its capillary, as shown in figure 11. During this operation the capillary is pressed into the bottom of the cup with one hand, so that the rubber ring makes a tight seal under the mercury, while the cocks are turned with the other hand.

The leveling bulb of the manometric apparatus is then placed in the medium position (see fig. 37, p. 272) about level with the bottom of the manometric chamber, and the cock connecting chamber and bulb is left open, so that the contents of the chamber are under slight negative pressure. Gas from the sample container is then admitted to the chamber until the volume is approximately either 1.5 or 35 cc., according to whether a micro or macro analysis is to be done. The volume can, with slight practice, be estimated with sufficient accuracy by the height of the mercury surface above the 2 or the 50-cc. mark. The amount of gas should be enough to give between 450 and 550 mm. pressure at the 2 or 50 cc. volume used for the analysis.

The gas sample can be similarly admitted from any other form of container provided with a three-way cock. The form of container shown in figure 9, made of two 500-cc. bottles, is convenient for respired air, of which it is desirable to use 35-cc. samples. During admission of a sample the container is hung from a hook beside the gas apparatus.

Admission of sample estimated by pressure

Sometimes it is desirable to take a smaller sample than one which will give about 500 mm. of pressure. This may be the case, for example, when air is subsequently to be admitted to burn hydrogen in the gas sample. Whenever it is desirable to set the size of the sample at an intermediate amount the following procedure can be used. It can also be used for measuring the usual samples if one has difficulty in admitting the desired amounts with approximate precision by the technique outlined above.

The apparatus is prepared and the p_o reading taken as above described. The sample container is then permitted to hang in position, with its cock closed and the tip of its capillary outlet immersed in the mercury of the cup of the chamber, while the mercury in the chamber is lowered till it is about 10 mm. above the 50 cc. mark. The cock between leveling bulb and chamber is then closed, and the leveling bulb is rested in the medium position, level with the bottom of the evacuated chamber. The tip of the capillary from the sample container is then pressed into the bottom of the cup, and the cock of the container is turned to connect container and chamber. Then the cock at the top of the chamber is carefully opened just long enough to admit enough gas to depress the mercury level in the chamber a little below the 50-cc. mark. The mercury in the manometer rises 20 to 40 mm. The cock leading to the manometric leveling bulb is now opened to admit enough mercury to the chamber to make the meniscus rise again about 10 mm. above the 50-cc. mark; then enough gas is again let in to force the mercury a little below the mark. This is repeated one, two, or three times, until the mercury in the manometer has risen to about the desired final level. During this procedure the tube of the sample container is held in position with the left hand, while with the right hand one alternately opens the cock at the top of the chamber to admit gas, then the cock from the leveling bulb to admit mercury. The procedure is in fact simple, and the admission of the entire sample by alternate turns of the two cocks requires less than a minute. Before the last admission of gas, the amount of mercury admitted is regulated from previous experience so that the subsequent admission of enough gas to press the mercury

down to the mark will raise the mercury column in the manometer to the desired height; e.g. the amount of mercury admitted before the last portion of gas may need to be only enough to raise the meniscus 5 mm., instead of 10 or 15, above the 50 cc. mark in the chamber.

Measurement of sample by pressure

After the sample has been admitted by either of the above procedures, the sample container is removed and the bore of the cock at the top of the chamber is filled with mercury from the cup (this cock is gas tight only when ***both bores are filled with mercury***). The mercury meniscus is then lowered below either the 2 cc. or the 50-cc. mark, and is brought back to the mark as above directed. The manometer reading p_1 is then taken, and the temperature of the chamber is read on the thermometer in the water jacket. The size of the gas sample is indicated by the pressure P_S, which the gas exerts at the chosen volume, either 2 or 50 cc.

$$P_S = p_1 - p_0$$

The *volume* of the gas sample, reduced to 0°, 760 mm., may be calculated from P_S and the temperature according to Equation 1 on page 124. However, the results of most analyses with this apparatus are calculated directly from the pressure measurements, so that calculation of volumes is unnecessary. The method for determining minute concentrations of CO_2 in air (p. 125) is an exception.

DETERMINATION OF OXYGEN AND CARBON DIOXIDE BY SIMPLE ABSORPTION. VAN SLYKE AND SENDROY (26)

The method here described is more rapid, but less exact, than those outlined later for manometric determination of CO_2 and O_2 in respiratory air. All the gas pressures are read with the mercury in the chamber at the 50-cc. mark. As in the Haldane analysis, the determination is accomplished by absorption of CO_2 and O_2, and the residual gas is determined as N_2. (It is of course a mixture of N_2 and about 1 per cent of argon, but in air analyses is conventionally reported as nitrogen.)

The relative accuracies of the method to be described and of the Haldane method may be indicated as follows. In the Haldane analysis the smallest division on the graduated scale of the burette measures 0.01 cc. or 1/1000 of the total volume, and the error to which the method is liable is that corresponding to an error of 0.2 or 0.3 of a scale division, or 0.02 to 0.03 per cent of the total gas. In the manometric analysis a scale division of 1 mm. on the manometer corresponds to 1/500 of the total gas sample, and an error of 0.2 to 0.3 of a scale division in the determination of any of the 3 gases involves

an error of 0.04 to 0.06 volume per cent, or twice that of the Haldane analysis. An occasional analysis may show an error of 0.10 volume per cent, but when duplicates are performed a practiced analyst can make certain of his result within 0.05 volume per cent.

The method here described is most simple in execution, and is sufficiently exact for many purposes, such as determination of the CO_2 in alveolar air for measurement of the CO_2 tension, and determination of O_2 in respiratory gas from a Tissot spirometer when the object is to determine oxygen consumption within 1 or 2 per cent, without precise estimation of the respiratory quotient.

When more accurate manometric results are needed, CO_2 and O_2, are determined by the methods later described in which the precision is increased, by isolation of the CO_2 from other gases, and by combustion of the O_2.

Reagents

1 N sodium hydroxide.

Thirty per cent sodium hyposulfite in 4 N potassium hydroxide. Fifteen grams of the hyposulfite, $Na_2S_2O_4$, are stirred up with 50 cc. of 4 N potassium hydroxide solution and filtered quickly through cotton into a 100 cc. flask containing enough paraffin oil to make a layer 1 or 2 cm. thick.

Neither of the above solutions needs to be freed of air for this analysis, since the solutions are to be shaken with the air sample at but little less than atmospheric pressure.

The anthraquinone beta-sulfonate catalyst is left out of the hyposulfite solution, because the intensely red color of the catalyst prevents reading the mercury meniscus when the solution is in the chamber. The analysis could be accomplished in 1 minute less time with the sulfonate catalyst present, but it appears usually to be preferable to use the water-clear solution of hyposulfite without the catalyst.

Measurement of sample

The sample of 30 to 35 cc. is admitted into the apparatus as described above on p. 108 and P_S is measured as described on p. 112 for "Measurement of sample by pressure."

$$P_S = p_1 - p_0.$$

Absorption of CO_2

One cubic centimeter of 1 N sodium hydroxide is measured accurately into the chamber from a stop-cock pipette in the manner shown in figure

52, page 344. The pipette bears a rubber ring on its tip, like the end of the capillary of the gas container in figure 11, and as in that figure the tip is pressed into the cup of the manometric chamber underneath a layer of mercury. During the admission of the alkali the manometric leveling bulb is at the medium level, with the mercury surface in the bulb at about the height of the 50-cc. mark on the chamber. The cock between leveling bulb and chamber is left open, so that the contents of the chamber are under slight negative pressure, and the chamber is about one-third full of mercury. After the alkali is admitted the chamber is shaken for two minutes so that the alkali will absorb the CO_2 from the gas sample. On account of the weight of mercury in the chamber the shaking is somewhat slower than in most analyses with this apparatus. During the shaking the cock between the chamber and its leveling bulb is left open. The 10 or 15 cc. of mercury in the chamber and the alkali solution over the mercury are thrown about the chamber in such a way that thorough contact between gas and liquid is obtained, and CO_2 absorption is completed in two minutes. It is usually in fact finished in the first minute.

After absorption is finished the manometer reading p_2 is taken with the meniscus of the *mercury* (not of the water solution) at the 50-cc. mark.

Absorption of oxygen with hyposulfite

After the p_2 reading has been taken the cock between the leveling bulb and the chamber is opened and 3.00 cc. of hyposulfite solution, accurately measured from a calibrated stop-cock pipette, are run into the chamber in the same manner in which the 1 cc. of sodium hydroxide was added. The absorption of oxygen is accomplished by shaking the chamber in the same manner as for CO_2 absorption, except that for oxygen three minutes instead of two are taken.

After the absorption is completed the meniscus of the *mercury* is brought to the 50-cc. mark and p_3 is read.

The gas is then ejected from the top of the chamber without loss of any of the solution (for technique for such ejection see page 279 of chapter VII), and p_4 is read with the 4 cc. of solution, but with no gas, in the chamber. The analysis is now complete.

Calculation

All quantities of gas measured are calculated in terms of the pressure exerted with the gas at 50 cc. volume and at the temperature of the P_S measurement. The sample is calculated as:

$$P_S = p_1 - p_0$$

The pressure of $O_2 + N_2$ is measured at 49 cc. volume. To calculate it for 50 cc. volume therefore it is necessary to multiply the observed pressure by 49/50, or 0.98.

$$P_{O_2 + N_2} = 0.98\ (p_2 - [p_0 + c])$$

The significance of the c correction will be discussed below.

The pressure of the CO_2 is calculated by subtracting the pressure of the $O_2 + N_2$ from that of the total sample.

$$P_{CO_2} = P_S - P_{O_2 + N_2}$$

The pressure of the N_2 at 46 cc. volume is measured as $p_3 - p_4$. At 50 cc. therefore one calculates:

$$P_{N_2} = 0.92\ (p_3 - p_4)$$

The oxygen is calculated by subtracting the N_2 from the $O_2 + N_2$

$$P_{O_2} = P_{O_2 + N_2} - P_{N_2}$$

The final results are calculated from the above data as follows.

$$\text{Per cent } CO_2 = \frac{100\ P_{CO_2}}{P_S}$$

$$\text{Per cent } O_2 = \frac{100\ P_{O_2}}{P_S}$$

$$\text{Per cent } N_2 = \frac{100\ P_{N_2}}{P_S}$$

Remarks on the calculation

As shown above, one must multiply the observed $O_2 + N_2$ and N_2 pressures by 0.98 and 0.92 respectively in order to calculate the pressures at 50-cc. volume from those at 49 and 46 cc. In practice the simplest way to make this calculation is to subtract 0.02 and 0.08 of their values from the observed pressures, $p_2 - [p_0 + c]$ and $p_3 - p_4$.

Example of calculation. Data from an analysis of laboratory air. $c =$ 1.4 mm. for the chamber used.

$$
\begin{array}{lll}
p_1 = 551.4 \text{ mm.} & & p_2 = 561.8 \text{ mm.} \\
p_0 = \underline{88.0} & & p_0 + c = \underline{89.4} \\
P_S = 463.4 & & P_{O_2 + N_2} \text{ at 49 cc.} = 472.4 \\
& & 0.02 \text{ of same} = \underline{9.4} \\
& & P_{O_2 + N_2} \text{ at 50 cc.} = 463.0
\end{array}
$$

$$
\begin{array}{r}
p_3 = 487.0 \text{ mm.} \\
p_4 = \underline{89.0} \\
P_{N_2} \text{ at 46 cc.} = 398.0 \\
0.08 \text{ of same} = \underline{31.8} \\
P_{N_2} \text{ at 50 cc.} = 366.2
\end{array}
$$

$$\text{Per cent } CO_2 = 100 \times \frac{463.4 - 463.0}{463.4} = 0.09$$

$$\text{Per cent } O_2 = 100 \times \frac{463.0 - 366.2}{463.4} = 20.89$$

$$\text{Per cent } N_2 = 100 \times \frac{366.2}{463.4} = 79.02$$

Determining the c correction

After the CO_2 has been absorbed the pressure exerted by the residual $O_2 + N_2$ at 49-cc. volume would be inexactly calculated as $p_2 - p_0$. The zero manometer reading without any gas present is, on account of the 1 cc. of NaOH solution present, slightly higher than the p_0 observed at the beginning of the analysis with neither gas nor solution in the chamber. The weight of the short column of water in the chamber presses on the mercury there and in consequence raises the height of the mercury column in the manometer tube required to hold the meniscus in the chamber at the 50-cc. mark. The c correction required for this effect varies somewhat with the shape of the bottom of the chamber and the consequent height of the column of 1 cc. of solution. It is, however, usually in the neighborhood of 1.5 mm.

The correction is determined as follows. The p_o point is determined, with the chamber free of both gas and visible amounts of water, as described on page 107. Then 1 cc. of 1 N sodium hydroxide is admitted, as described for absorption of CO_2, the chamber is evacuated till the mercury falls to the 50-cc. mark, and the air is extracted from the solution by shaking the latter one minute. The extracted small bubble of

gas is ejected from the top of the chamber without loss of solution, as described on page 279. The chamber is again evacuated and the mercury meniscus is brought to the 50 cc. mark. The manometer is again read. The difference between the two readings is the c correction. To determine c within 0.1 mm. one makes several check readings of p_o, and also several of $p_o + c$ with the 1 cc. of solution in the chamber. Once determined, the c correction serves for all analyses in which the same chamber is used.

Corrections for temperature changes

It is desirable to avoid temperature changes in the water jacket of the apparatus during an analysis. Changes exceeding 0.1° can usually be avoided by the precautions outlined on page 108.

The temperature is observed on the thermometer in the water jacket, and if changes do occur the following corrections are applied. If the temperature t_2 at the p_2 reading differs by more than 0.1° from the temperature t_0 at the p_0 reading, the corrected formula given below is used for calculating $P_{O_2+N_2}$. In this formula the term 1.3 $(t_0 - t_2)$ is introduced to correct for the effect of vapor pressure change caused by temperature change between the p_0 and p_2 readings. The factor T_0/T_2 corrects for changes in pressure of the gases other than the water vapor.

If the temperature at the p_3 reading differs from that at the p_0 reading, the corrected formula below for calculating P_{N_2} is used.

T_0, T_2, and T_3 represent absolute temperature (= temperature centigrade + 273°) observed in the apparatus at the p_0, p_2, and p_3 readings, respectively.

The p_1 reading is taken so quickly after the p_0, and the p_4 after the p_3, that there is practically no opportunity for changes in temperature between the p_0 and p_1 or between the p_3 and p_4 readings.

$$\text{Corrected } P_{O_2+N_2} \quad 0.98\ (p_2 + 1.3\ [t_0 - t_2] - [p_0 + c]) \times T_0$$

$$\text{Corrected } P_{N_2} = 0.92\ (p_3 - p_4) \times T_0$$

Necessity for accuracy in measurement of volumes of alkali and hyposulfite solutions added

The accuracy with which the volumes 49 and 46 cc. are defined, at which the pressures of residual gases are measured after absorption of CO_2 and O_2

respectively, is determined by the accuracy with which the 1 and 3-cc. portions of alkali and hyposulfite solution are measured into the chamber. An error of 0.01 cc. in the measurement of either solution would cause an error of 1 part in 5000 in the volume of gas space in the chamber at which the pressure is measured. With calibrated stop-cock pipettes, however, it is simple to make the deliveries of solution into the chamber with errors less than 0.01 cc.

Similar accuracy in the calibration of the exact total gas volume held above the 50-cc. mark is not necessary. Any error in this calibration will have so nearly a proportional effect on pressure measurements at 46 cc., that an error of 0.1 cc. in the 50-cc. calibration is required to affect oxygen results by 1 part in 5,000.

NITROGEN IN AIR, OR AS IMPURITY IN O_2 OR CO_2 GAS

In some cases, as in the lung volume determinations by the nitrogen dilution method described in chapter VI, the nitrogen content of the air is the only figure desired. In this case the analysis is simplified by absorbing the CO_2 and O_2 together by the alkaline hyposulfite.

The sample is measured as in the preceding analysis.

$$P_S = p_2 - p_3$$

Then 3 cc. of the hyposulfite solution are introduced into the chamber, as above described, and the O_2 and CO_2 are absorbed together by three minutes shaking. Reading p_2 is taken with the mercury meniscus at the 50-cc. mark. The gases are then ejected without loss of solution and reading p_3 is taken.

All readings being taken with the mercury meniscus in the chamber at the 50 cc. mark, the calculations resemble those of the proceding analysis.

$$P_{N_2} = 0.94\ (p_2 - p_3)$$

$$\text{Per cent } N_2 = 100 \times \frac{P_{N_2}}{P_S}$$

$$\text{Per cent } CO_2 + O_2 = 100 - \text{per cent } N_2.$$

In case one wishes to determine traces of N_2 as impurity in CO_2 or O_2 the unabsorbed N_2 is measured by its pressure at 2 or 0.5-cc. volume. In such analyses it is desirable to use gas-free hyposulfite solution. P_S is measured as above described. Then the O_2 and CO_2 are ab-

sorbed with air-free hyposulfite, and p_2 is read with the unabsorbed gas at either 2 or 0.5 cc. volume. The gas is then ejected without loss of solution (for technique see p. 279 in chapter VII), and p_3 is read, with the chamber free of gas and the solution meniscus at the same mark as for the p_2 reading.

$$P_{N_2} = p_2 - p_3$$

$$\text{Per cent } N_2 = 100 \times \frac{a}{A} \times \frac{P_{N_2}}{P_S}$$

a = the small volume, 0.5 or 2 cc., at which P_{N2} is measured, and A is the total chamber volume, 50 cc. in the usual chamber, at which P_S is measured. For chambers of the ordinary dimensions, with A = 50 cc. and a = 0.5 or 2 cc., the calculation simplifies to the following.

$$\text{Per cent } N_2 = \frac{P_{N_2}}{-} \text{ when } a = 0.5 \text{ cc. or}$$

$$\text{Per cent } N_2 = \frac{4\,P_{N_2}}{P_S} \text{ when } a = 2 \text{ cc.}$$

CARBON DIOXIDE IN RESPIRATORY AIR BY THE ISOLATION METHOD. VAN SLYKE, SENDROY AND LIU (29)

Principle. The CO_2 is first isolated from other gases by its absorption with alkali solution in the chamber of the manometric apparatus. The other gases are then ejected, the absorbed CO_2 is set free by acid, and is determined as in blood analyses. By this procedure the CO_2 in any desired volume of gas can be absorbed, and then set free and determined by the pressure it exerts at 2 or 0.5 cc. volume. The CO_2 content of atmospheric air has thus been determined to 0.0001 volume per cent, or 0.003 the amount of CO_2 ordinarily present in the atmosphere. Such precision with apparatus modeled on the usual principles of gas analysis can be obtained only with elaborate precautions. The method also serves for determination of CO_2 in samples of expired or alveolar air, where the CO_2 content runs from 6 to 2 per cent. In this case the usual accuracy is 0.02 to 0.03 volume per cent, about the same as with the usual type of Haldane apparatus. In analysis of gases with more than 15 per cent of CO_2 the method described on p. 112 is preferable, unless it is desirable to use minimal samples. In that case the micro form of the method here described can be used, with samples of only 1.5 cc.

Reagents

5 N sodium hydroxide, approximate. Described on page 233.

1 N hydrochloric acid, approximate. 83 cc. of concentrated hydrochloric acid of 1.19 specific gravity diluted to a liter.

0.1 N sodium hydroxide, approximate, of minimal CO_2 content. 6 cc. of the saturated NaOH solution, described on page 29, are pipetted into 1 liter of water, which has been freed of CO_2 by adding a drop of concentrated hydrochloric acid and boiling. About 1 cc. of 1 per cent alizarin red solution is added. The 0.1 N alkali solution is immediately poured into 50-cc. flasks or bottles closed with paraffined corks or vaselined glass stoppers. After one of these flasks has been opened to use part of the solution, the residue is thrown away.

Apparatus

The only special apparatus besides the manometric is a 25 cc. burette for holding CO_2-free NaOH solution. The tip of the burette must be long enough to fit into the cup of the manometric chamber as shown in figure 52, page 344, and is provided with a rubber ring, like the pipette in that figure. The top of the burette is protected from air by a soda lime tube. When the burette is not being used the outlet is kept immersed in mercury to prevent absorption of CO_2 by the drop of alkali at the tip.

Introduction and measurement of sample

Macro samples. Before the gas sample is admitted to the chamber one estimates the approximate pressure in millimeters which a sample of desirable size will exert at 50 cc. volume. A sample of such size will contain 0.5 to 1.0 cc. of CO_2, which will give a pressure of 200 to 400 mm. at 2 cc. volume. A simple rule to calculate the pressure which a sample of desired size will exert at 50 cc. is to divide 1200 by the expected percentage of CO_2 in the gas. E.g. if alveolar air, with probably 6 per cent CO_2 is analyzed, a sample is taken which will give a P_S of $\frac{1200}{6} = 200$ mm. pressure at 50 cc. The CO_2 in this sample will then exert $0.06 \times 200 = 12$ mm. pressure at 50 cc., and 25 times as much, or 300 mm. at 2 cc. when P_{CO_2} is determined in the final measurement. When expired air from a Tissot spirometer, with a CO_2 content of usually about 4 per cent is analyzed, one takes sufficient sample to give a P_S of about 300 mm. If gas of less than 1.5 per cent CO_2 content is analyzed as large a sample as possible is taken, enough to give a P_S of 500 mm.

Before the sample is admitted the chamber is washed with acidulated water, which is ejected by slow admission of mercury, and the p_0 read-

ing is taken as described on p. 107. The sample of desired size is then measured as described above for "Admission of sample estimated by pressure," and the p_1 reading is taken with the mercury meniscus at the 50 cc. mark.

$$P_S = p_1 - p_0$$

Micro samples. A sample of about 1.5 cc. volume at atmospheric pressure is admitted as described above for "Admission of sample estimated by volume." The readings of p_0 and p_1 in this case are taken with the mercury in the chamber at the 2 instead of the 50 cc. mark.

Absorption of CO_2 from gas sample

After the gas sample has been measured 3.00 cc. of the CO_2-free 0.1 N sodium hydroxide solution are admitted into the chamber from the soda-lime guarded burette as shown in figure 52, page 344. Before inserting the burette tip into the cup of the chamber 0.5 cc. of the solution is wasted, in order to remove from the tip the drop which has absorbed CO_2 from the air. During admission of the alkali, the cock between the manometric chamber and its leveling bulb is left open, while the bulb is at the level shown in figure 37, p. 272, so that the contents of the chamber are under slight negative pressure.

After admission of the alkali the mercury in the chamber is lowered until only the lower third of the chamber is filled with the metal. The chamber is then shaken rather slowly for two minutes. This causes complete absorption of the CO_2 by the alkali, which is thrown about on top of the mercury in such a way that it comes into thorough contact with the gas.

The residual gases are then ejected by the technique described on page 279. It is not essential that the last few cubic millimeters of gas be ejected, but it is essential that none of the alkali solution rise into the cup. The ejection of gas is therefore stopped when the alkali solution has entered the bore of the stop-cock.

Determination of the absorbed CO_2

After ejection of unabsorbed gases 1 cc. of the 1 N hydrochloric acid is placed in the cup, and 0.5 cc. is run into the chamber. The CO_2 is now determined as described for blood analyses on pages 283–287 of chapter VII. The reading of the gas pressure p_2 is taken with 2 cc. gas volume, unless so little CO_2 is present that the P_{CO_2} at 2 cc. volume is less than 100 mm. In this case the reading is taken with the gas at 0.5 cc. volume.

The CO_2 is absorbed with 0.3 cc. of 5 N sodium hydroxide solution, as described on page 284, and reading p_3 is taken, with the same gas volume as at the p_2 reading. The alizarin indicator serves to show that the entire solution in the chamber turns alkaline.

A ***blank analysis*** is performed, in which no gas is admitted to the chamber. The pressure fall observed when the 5 N sodium hydroxide is added is the c correction. It should not exceed 4 to 6 mm. with the gas at 2 cc. volume if the 0.1 N alkali solution has been prepared and handled with the above outlined precautions to minimize its CO_2 content.

$$P_{CO_2} = p_2 - p_3 - c.$$

Calculations

The CO_2 content of the gas analyzed is calculated as:

$$\text{Volume per cent } CO_2 = \frac{P_{CO_2}}{P_S} \times \textit{factor}$$

the factor being taken from table 13.

Corrections for calibration errors of chamber and for effect of measuring P_S over a mercury meniscus. If a, the volume at which P_S is measured, is other than the 50 or 2 cc. assumed in calculating the factors of table 13, the observed P_S will have to be multiplied by the correction factor $a/2$ or $a/50$ to obtain the exact P_S for use with the factors of table 13. Similarly, if the volume at which P_{CO_2} is measured is other than the assumed 0.5 or 2.0 cc. the observed P_{CO_2} will require multiplication by $a/0.5$ or $a/2$ in order to obtain the exact P_{CO_2} for use with the factors of table 13.

In a well calibrated chamber the deviations from the assumed volumes will be negligible, except for P_S values, measured in the micro determinations, over a mercury meniscus at the 2 cc. mark. The chambers are calibrated for gas measurements over water menisci, and, as shown on page 19 of chapter I, the gas volume over a mercury meniscus at a given mark is greater than over a water meniscus at the same mark. If the bore of the chamber at the 2 cc. mark is 4 mm., as is generally the case, figure 4 (p. 20) indicates that the gas volume over a mercury meniscus at that mark will be 0.012 cc. greater than over a water meniscus. The value of P_S measured at a 2-cc. mark exact for a water meniscus will then require multiplication by the correction factor $\frac{2.012}{2.000} = 1.006$, when the measurement is made over a mercury meniscus.

In general, if a is the supposed gas volume (0.5, 2.0, or 50 cc.) held by the chamber over a water meniscus at a given mark, a' is the actual volume above a water meniscus found in checking the calibration, and c_a is the increase in gas volume measurement that results from changing from a water meniscus to a mercury meniscus, then the observed pressure, P_S or P_{CO_2} must be cor-

TABLE 13

FACTORS FOR CALCULATING CO_2 CONTENT OF A GAS. VAN SLYKE AND SENDROY (26)

TEMPERATURE	FACTORS WHEN SAMPLE PRESSURE IS TAKEN AT 50-CC. VOLUME		FACTORS WHEN SAMPLE PRESSURE IS TAKEN AT 2-CC. VOLUME	
	P_{CO_2} is measured with gas at 2-cc. volume	P_{CO_2} is measured with gas at 0.5-cc. volume	P_{CO_2} is measured with gas at 2-cc. volume	P_{CO_2} is measured with gas at 0.5-cc. volume
°C	*factor*	*factor*	*factor*	*factor*
15	4.393	1.120	109.8	27.98
16	83	17	9.6	92
17	73	15	9.3	87
18	65	13	9.1	82
19	58	12	9.0	77
20	51	10	8.8	72
21	44	08	8.6	68
22	37	06	8.4	63
23	30	04	8.3	59
24	24	02	8.1	54
25	18	00	7.9	50
26	12	1.099	7.8	47
27	06	97	7.6	44
28	00	96	7.5	41
29	4.295	95	7.4	38
30	91	94	7.3	34
31	86	93	7.2	31
32	81	92	7.0	27
33	76	91	6.9	24
34	71	90	6.8	20

rected by multiplication by $\frac{a'}{a}$ if the pressure is observed with the gas over an aqueous meniscus, and by $\frac{a' + c_a}{a}$ when the pressure is observed with the gas over a mercury meniscus. In the CO_2 determinations in gas mixtures

above described, c is significant only in the measurement of P_S values at 2 cc. for micro samples. For P_S measured with the gas at 50 cc. volume, as is the case except when micro samples are taken, the difference between mercury and water menisci is only enough to affect P_S by about 1 part per 1000, and may ordinarily be neglected. P_{CO_2} values in this analysis are all measured over water menisci, so that the c_a correction does not apply to P_{CO_2}.

Example. If a' measured over a water meniscus is found by the usual calibration from weight of water delivered to be 2.010 instead of 2.000 cc., and the bore of the chamber at the 2 cc. mark is 4 mm., so that c_a = 0.012 cc. (see fig. 4, p. 20), then the correction factor by which observed micro P_S values must be multiplied will be

$$\frac{a' + c_a}{a} = \frac{2.010 + 0.012}{2} = 1.011.$$

The correction factor for P_{CO_2} (if P_{CO_2} is also measured at the 2 cc. mark) will be $\frac{2.010}{2.000}$ = 1.005. With these correction factors, the calculation formula would become:

$$\text{Volume per cent } CO_2 = \frac{P_{CO_2} \times 1.005}{P_S \times 1.011} \times \textit{factor} = \frac{P_{CO_2}}{P_S} \times 0.994 \times \textit{factor}.$$

The correction factor 0.994 would be constant for this chamber and type of analysis.

The *factors in table 13* are calculated as follows. V_{sample} and V_{CO_2} indicate respectively the volumes at 0° and 760 mm. of the gas sample and of the CO_2 in it. V_m indicates the volume, either 50 or 2 cc., at which P_S is measured. From the gas laws we have:

$$V_{sample} = V_m \times \frac{P_S}{760} \times \frac{1}{1 + 0.00384\, t} \tag{1}$$

From equation 2 on page 282 in chapter VII we have:

$$V_{CO_2} = a \times \frac{P_{CO_2}}{760} \times \frac{1}{1 + 0.00384\, t} \times i \left(1 + \frac{S\, \alpha'}{A - S}\right) \tag{2}$$

The volume per cent of CO_2 in the sample is $\frac{100\, V_{CO_2}}{V_{sample}}$. This is calculated by dividing the lower of the above equations by the upper, and multiplying by 100.

$$\text{Per cent } CO_2 = \frac{P_{CO_2}}{n} \times \frac{100\, i\, a \left(1 + \frac{S}{A - S}\right)}{V_n}. \tag{3}$$

Since the value of S, the cubic centimeters of solution extracted, is constant at 3.5 cc., and A is 50 cc., the above equation simplifies, for these conditions, to

$$\text{Per cent } CO_2 = \frac{P_{CO_2}}{P_S} \times \frac{100\, i\, a}{V_m} (1 + 0.0753\, \alpha') \tag{4}$$

A slight correction is required for the effect of the sodium chloride present in 0.085 *M* concentration on the solubility of CO_2. The α' of CO_2 in this solution is 98.3 per cent as great as in water (2). Hence the coefficient of α' is multiplied by 0.983. We thus obtain

$$\text{Per cent } CO_2 = \frac{P_{CO_2}}{P_S} \times f$$

where $f = \frac{100\ i\ a}{V_m}(1 + 0.074\ \alpha')$

The value of i, the factor correcting for reabsorption of CO_2, is 1.017 when a, the volume at which P_{CO_2} is measured, is 2 cc. When a is 0.5 cc. i is 1.037.

PRECISE DETERMINATION OF CO_2 IN ATMOSPHERIC AIR. VAN SLYKE AND SENDROY (26)

If the CO_2 in 35 cc. of air containing 0.03 per cent of CO_2 is measured at 0.5 cc. volume it will give about 16 mm. of pressure, and an error of 1 mm. will make one of 0.002 volume per cent of CO_2 in the result. This is about the limit of accuracy for the procedure as above described. However, one is not limited to the volume of sample that can be measured in one portion in the chamber of the apparatus. Successive portions of air can be run into the chamber and the CO_2 from all of them absorbed by one portion of alkali, so that the accuracy of the analysis can, if desired, be made to approach 0.0001 per cent of an atmosphere.

When the accuracy obtainable from a large air sample is desired the sample is measured by volume in a container of the type shown in figure 10, I, 1. The container, of for example 250 cc. volume, is calibrated by weighing it first empty, except for a film of water on the inner wall, and then with the bulb between the two cocks filled with water (see chapter I). The container, of which the inner walls should be moist, is at first filled with mercury, which is then entirely displaced by a sample of the air, the mercury being withdrawn as far as the lower cock. The container is then connected to the manometric chamber as shown in figure 12 by a flexible rubber tube of about 2-mm. bore and just sufficient length to permit the chamber to be shaken without disturbing the container. The connecting tube is then filled with mercury from the chamber, and about 35 cc. of the sample are run into the chamber. Three cubic centimeters of the 0.1 N sodium hydroxide are then admitted to the chamber as above described, and the CO_2 in the air portion is absorbed by shaking slowly for two minutes, with 10 or 15 cc. of mercury in the chamber. The unabsorbed gas, except for a small bubble, is then ejected. Then

another portion of the sample is admitted and its CO_2 is absorbed in the same manner. This procedure is repeated until all the gas from the calibrated container has passed through the chamber and been shaken with the alkali. When the last portion of gas is run into the chamber a

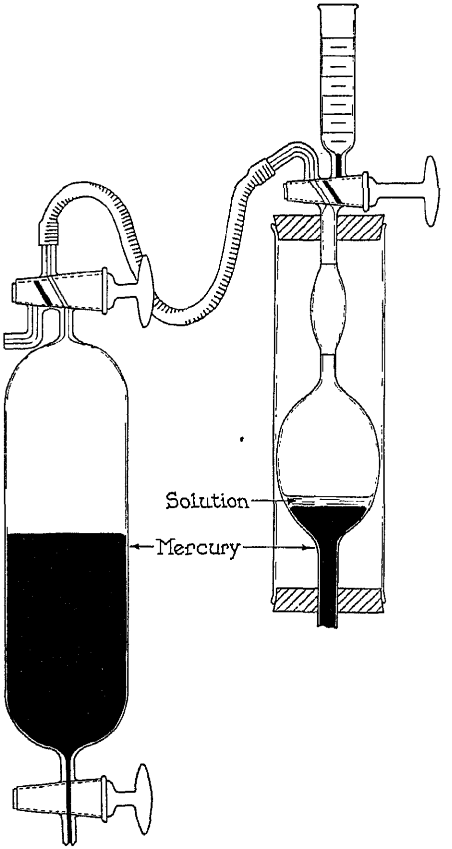


FIG. 12. Transferring gas sample from calibrated container to chamber of manometric apparatus.

little mercury from the container is permitted to follow and fill the bore of the cock of the chamber.

After the CO_2 from the last portion of air has been absorbed, the unabsorbed air is ejected and 0.5 cc. of I N hydrochloric acid is admitted. The CO_2 is extracted from solution and P_{CO_2} is measured as directed for the preceding analysis.

Calculation

$$\text{Per cent } CO_2 = 100\ V_{CO_2}$$

V_{CO_2} represents the cubic centimeters of CO_2, reduced to 0°, 760 mm., and V_S represents the volume of the sample in the same units.

The value of 100 V_{CO_2} is obtained by multiplying P_{CO_2} by the proper factor from table 14, p. 128.

V_S is calculated by multiplying the observed volume of the sample (the volume content of the calibrated container) by the proper factor from table 15, p. 129, to reduce the volume to standard conditions.

The calculation may therefore be expressed as

$$\text{Per cent } CO_2 = \frac{f_1\ P_{CO_2}}{f_2\ C}$$

where f_1 is the factor from table 14, f_2 the factor from table 15, and C is the capacity in cubic centimeters of the container in which the gas sample was measured.

OXYGEN IN AIR BY COMBUSTION WITH HYDROGEN. VAN SLYKE AND HANKE (22)

Principle. An excess of hydrogen is added to the air and the mixture is ignited with a platinum spiral. The principle has been more frequently used for determination of hydrogen, but it is equally simple for oxygen. The manometric apparatus is especially convenient for determinations of oxygen and hydrogen by combustion, because it obviates the necessity of diluting the mixtures with inert gas to avoid too vigorous explosions. Explosive mixtures are simply attenuated by putting them under reduced pressure when they are burned.

The reaction, $2\ H_2 + O_2 = 2\ H_2O$, has for oxygen determination the advantage that the shrinkage in gas that results from the combustion is three times the volume of the oxygen. In consequence the accuracy of the determination is increased, and oxygen determinations in the ordinary form of manometric apparatus can be done with an error of usually not more than 0.02 or 0.03 volume per cent.

Apparatus

Figure 13 illustrates the construction and manner of attachment of the combustion chamber to the manometric chamber of Van Slyke and Neill.

The combustion chamber differs only in details from that used with the Haldane gas analyzer. It consists of a heavy walled Pyrex glass cylinder, of about 100 mm. length and 32 mm. diameter, and therefore about 75 cc. capacity. The mouth of the cylinder is expanded slightly so that it will fit the rubber stopper. The glass here is also thickened somewhat and a slight

TABLE 14

Factors by Which P_{CO_2} is Multiplied to Calculate 100 V_{CO_2}. Van Slyke and Sendroy (26)

(S = 3.5 cc.)

Temperature	Factor when P_{CO_2} is measured with gas at 2-cc. volume	Factor when P_{CO_2} is measured with gas at 0.5-cc. volume
°C.		
15	0.2735	0.0697
16	19	93
17	04	89
18	0.2690	86
19	75	82
20	62	78
21	48	75
22	34	71
23	20	68
24	07	65
25	0.2594	61
26	81	58
27	69	55
28	57	52
29	45	49
30	33	46
31	22	43
32	11	40
33	00	37
34	0.2489	34

rim is put on the bottom, so that the greased stopper can be forced in without breaking the glass.

Through the stopper pass three glass tubes. One is connected by about 1 meter of fresh, thick-walled "nitrometer" tubing of red rubber with the leveling bulb shown in figure 13.[6] Into the upper ends of the other two are

[6] The rubber tubing must be so impervious to air that when the leveling bulb is lowered and a partial vacuum in the combustion chamber is produced, no air leaks through this

TABLE 15

Factors for Reducing Volume of Moist Gas to Volume Occupied by Dry Gas at 0°, 760 mm.

Observed Barometric Reading, Uncorrected for Temperature	15°	16°	17°	18°	19°	20°	21°	22°	23°	24°	25°	26°	27°	28°	29°	30°	31°	32°
700	0.855	851	847	842	838	834	829	825	821	816	812	807	802	797	793	788	783	778
702	857	853	849	845	840	836	832	827	823	818	814	809	805	800	795	790	785	780
704	860	856	852	847	843	839	834	830	825	821	816	812	807	802	797	792	787	783
706	862	858	854	850	845	841	837	832	828	823	819	814	810	804	800	795	790	785
708	865	861	856	852	848	843	839	834	830	825	821	816	812	807	802	797	792	787
710	867	863	859	855	850	846	842	837	833	828	824	819	814	809	804	799	795	790
712	870	866	861	857	853	848	844	839	836	830	826	821	817	812	807	802	797	792
714	872	868	864	859	855	851	846	842	837	833	828	824	819	814	809	804	799	794
716	875	871	866	862	858	853	849	844	840	835	831	826	822	816	812	807	802	797
718	877	873	869	864	860	856	851	847	842	838	833	828	824	819	814	809	804	799
720	880	876	871	867	863	858	854	849	845	840	836	831	826	821	816	812	807	802
722	882	878	874	869	865	861	856	852	847	843	838	833	829	824	819	814	809	804
724	885	880	876	872	867	863	858	854	849	845	840	835	831	826	821	816	811	806
726	887	883	879	874	870	866	861	856	852	847	843	838	833	829	824	818	813	808
728	890	886	881	877	872	868	863	859	854	850	845	840	836	831	826	821	816	811
730	892	888	884	879	875	871	866	861	857	852	847	843	838	833	828	823	818	813
732	895	890	886	882	877	873	868	864	859	854	850	845	840	836	831	825	820	815
734	897	893	889	884	880	875	871	866	862	857	852	847	843	838	833	828	823	818
736	900	895	891	887	882	878	873	869	864	859	855	850	845	840	835	830	825	820
738	902	898	894	889	885	880	876	871	866	862	857	852	848	843	838	833	828	822
740	905	900	896	892	887	883	878	874	869	864	860	855	850	845	840	835	830	825
742	907	903	898	894	890	885	881	876	871	867	862	857	852	847	842	837	832	827
744	910	906	901	897	892	888	883	878	874	869	864	859	855	850	845	840	834	829
746	912	908	903	899	895	890	886	881	876	872	867	862	857	852	847	842	837	832
748	915	910	906	901	897	892	888	883	879	874	869	864	860	854	850	845	839	834
750	917	913	908	904	900	895	890	886	881	876	872	867	862	857	852	847	842	837
752	920	915	911	906	902	897	893	888	883	879	874	869	864	859	854	849	844	839
754	922	918	913	909	904	900	895	891	886	881	876	872	867	862	857	852	846	841
756	925	920	916	911	907	902	898	893	888	883	879	874	869	864	859	854	849	844
758	927	923	918	914	909	905	900	896	891	886	881	876	872	866	861	856	851	846
760	930	925	921	916	912	907	902	898	893	888	883	879	874	869	864	859	854	848
762	932	928	923	919	914	910	905	900	896	891	886	881	876	871	866	861	856	851
764	936	930	926	921	916	912	907	903	898	893	888	884	879	874	869	864	858	853
766	937	933	928	924	919	915	910	905	900	896	891	886	881	876	871	866	861	855
768	940	935	931	926	922	917	912	908	903	898	893	888	883	878	873	868	863	858
770	942	938	933	928	924	919	915	910	905	901	896	891	886	881	876	871	865	860
772	945	940	936	931	926	922	917	912	908	903	898	893	888	883	878	873	868	862
774	947	943	938	933	929	924	920	915	910	905	901	896	891	886	880	875	870	865
776	950	945	941	936	931	927	922	917	912	908	903	898	893	888	883	878	872	867
778	952	948	943	938	934	929	924	920	915	910	905	900	895	890	885	880	875	869
780	955	950	945	941	936	932	927	922	917	912	908	903	898	892	887	882	877	872

sealed short heavy platinum wires, which are joined within the chamber by a thin platinum wire (no. 26 or 28) about 5 cm. long, bend into three spirals. After the platinum wires are sealed into the glass, each tube is filled with melted Wood's metal, and a strong copper wire is inserted into the cooling metal. This device (suggested by Sendroy) enables one to economize on platinum, and to use strong and heavy copper wires for connections below the tubes with the battery wires. Electricity to heat the platinum spiral is provided by a battery of two or three dry cells.

To avoid danger from flying glass in case a miscalculation of the mixture burned should cause a highly explosive one to be ignited, it is well to wrap a layer of wire guaze about the combustion chamber, and bind it with wire.

TABLE 15A

FACTORS FOR CONVERTING PRESSURES AT 50-CC. VOLUME AND t° INTO VOLUMES AT 760 MM. AND 0°.

TEMPERATURE	f_2	TEMPERATURE	f_2
°C.		°C.	
15	0.06220	25	0.06003
16	6188	26	5982
17	76	27	61
18	53	28	40
19	32	29	20
20	0.06109	30	0.05899
21	6089	31	79
22	66	32	59
23	45	33	39
24	24	34	19

The ends of the capillaries of the combustion and reaction chambers are ground flat so that when they meet there is a minimum of dead space. The rubber tube connecting the capillaries from the two chambers is new, heavy-walled grayish red "nitrometer" tubing with a small enough bore so that it grips the glass tubes firmly.

rubber tube and rises into the chamber. The heavy walled nitrometer tubing of grayish red rubber meets these requirements, but after some months becomes permeable to gas and must be changed. Before the tubing is attached its bore is cleaned and lined at the ends with a thin layer of vaseline or stop-cock grease, with which the glass end over which the rubber is to be drawn is also coated. In case rubber tubing sufficiently impermeable to gas is not available, a Shohl air trap, shown in figure 28 on page 237 of chapter VII, may be sealed to the glass tube below the combustion chamber.

In order to ensure joints which will not leak under reduced pressure the piece of nitrometer tubing and the rubber stopper used on the combustion chamber are cleaned and boiled with dilute alkali before use, and both rubber and glass surfaces are covered with thin layers of grease before they are joined. The stopper is held tightly in place by wires. It is unnecessary to bind with wire or otherwise the rubber tube shown in figure 13 connecting

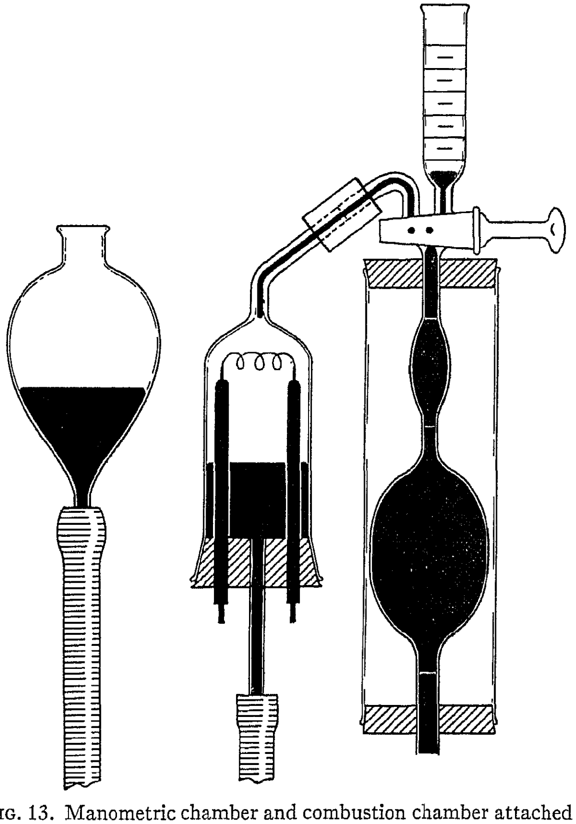


FIG. 13. Manometric chamber and combustion chamber attached

the two chambers. Binding is undesirable because it makes the rubber tube spread slightly and thereby increases the dead space between the ends of the glass capillaries. The combustion chamber is supported at such a level that its capillary meets that of the Van Slyke-Neill chamber exactly. With these precautions the joint will not leak when the pressure in the combustion chamber is reduced to 100 mm. (leveling bulb lowered 660 mm.), which is the maximum evacuation used.

The leveling bulb of the combustion chamber is conveniently hung by a hook from the links of a chain, the lowest link of which does not permit the surface of the mercury in the bulb to fall by more than 660 mm. below the floor of the combustion chamber. (The type of chain used for supporting window sash is convenient.)

The combustion chamber can be easily attached or detached in one minute, and there is no reason why this analysis should not form one step in a series of analyses of which the others are done without the combustion chamber.

In some Van Slyke-Neill reaction chambers the curved outlet capillary approaches so near to the end of the cock that either the bend of the capillary must be changed or the end of the cock ground away to make room for the heavy rubber tube used to connect the manometric chamber with the combustion chamber.

Reagents

The only reagent required is hydrogen. No harm is done if it contains a little nitrogen as impurity, but it must be free from oxygen. The hydrogen is tested for oxygen by submitting a sample to combustion, without admixture of other gases, in the manner described below. It can be kept in a container of the form shown in figure 9. Pyrogallol, covered by oil on the surface exposed to air, can be used as fluid in the container if the hydrogen is found to contain oxygen.

Admission and measurement of sample

Before the sample of gas is admitted the combustion chamber is attached to the manometric chamber as shown in figure 13, and mercury is drawn over into the manometric chamber from the combustion chamber, sweeping out air from the connections. To remove traces of air which are likely to be trapped between the ends of the connecting capillaries, the mercury in the manometric chamber is lowered, and the cock at the top of that chamber is opened to connect quickly with the combustion chamber. The rush of 2 or 3 cc. of mercury through the connections into the evacuated manometric chamber sweeps out any traces of air that may have remained in the connections.

The sample is admitted as described on page 108. Before the first of a series of analyses the chamber is washed with acidulated water, in order to make certain that no alkali is present to combine with CO_2 in the gas sample. Between the analyses of a series of oxygen determinations it is not necessary to wash the chamber. The sample should pref-

erably be 30 to 35 cc., enough to give a pressure of about 500 mm. at 50 cc. volume. Micro analyses can be done, with an accuracy of 0.2 volume per cent, on samples of 1.5 cc., with pressure measurements at 2 cc. volume, but economy of air samples is seldom necessary. The pressure of the sample is calculated as usual:

$$P_S = p_1 - p_0$$

After the sample is measured it is transferred to the combustion chamber. For this purpose the leveling bulb of the combustion chamber is lowered below the level of that chamber and the cock at the top of the manometric chamber is opened to connect the two chambers. Mercury is then admitted into the bottom of the manometric chamber until all the gas, followed by a little mercury, has been driven over into the combustion chamber. Thus the transfer of gas is accomplished without at any time putting the gas in the manometric chamber under positive pressure, which would be undesirable, since it might cause measurable leakage of gas out of the cock at the top of the chamber. (The cock is designed to hold against complete vacuum when its bores are filled with mercury, but can not be trusted always to hold against positive pressure inside the chamber.)

Addition of hydrogen

For air containing 21 per cent of oxygen, hydrogen sufficient at 50 cc. to give 42 per cent as much pressure as the P_S of the sample is needed. In general, for all air analyses, it is convenient to add enough hydrogen to give a pressure equal to approximately half of the measured P_S. The hydrogen is added in the manner described on page 111 for "admission of samples estimated by pressure."

The manometer reading p_2 with the hydrogen in the chamber is taken in the same manner as the p_1 reading with the sample in the chamber. The pressure P_{H_2} of the hydrogen is

$$P_{H_2} = p_2 - p_0$$

The measured hydrogen is all run over into the combustion chamber, and then one-half of the mixture of the hydrogen and air is returned to the manometric chamber.

Combustion

In the presence of excess hydrogen, as added in this analysis, gas mixtures will explode when the partial pressure of the oxygen exceeds 50

mm., but not if it is less. Moderate explosions, really only ignition by flash without serious force, occur if the 50-mm. limit is moderately exceeded, but explosions severe enough to threaten the glass chamber do not result unless the oxygen tension approaches twice the 50-mm. limit. In air under atmospheric pressure the oxygen tension is about 155 mm., and it is reduced to about 100 mm. by dilution with 0.5 volume of hydrogen. By lowering the total pressure on the gas mixture from 1 atmosphere to 0.5 atmosphere one therefore will reduce the oxygen tension to the desired 50 mm.

This is accomplished by lowering the leveling bulb of the combustion chamber so that the level of its mercury is 400 mm. below the floor of that chamber. The portion of about 25 cc. of gas in the combustion chamber will expand as the result of the partial vacuum until it fills about two-thirds of the 75 cc. chamber. The wire is then heated and kept glowing for thirty seconds. Then the current is stopped and another thirty seconds is allowed for the wire to cool. The remainder of the sample is then passed from the manometric chamber into the combustion chamber. (If the wire were not allowed to cool first, the incoming stream of gas might be ignited.)

The leveling bulb of the combustion chamber is adjusted at such a level that the mercury sinks to within 10 or 15 mm. of the bottom of the chamber, and the ignition is repeated.

The ignited gases are then returned to the manometric chamber, and are allowed to rest there two minutes to come to the temperature of that chamber. The manometer reading p_3 is then taken, with the gas at 50 cc. volume. The pressure of the residual gas, P_R, is then calculated as

$$P_R = p_3 - p_0$$

If there is any doubt that combustion has been complete, after the p_3 reading the gas, instead of being ejected, is returned to the combustion chamber and again ignited for 30 seconds, and the p_3 reading is repeated.

If there has been any temperature change in the water jacket of the manometric chamber since the first p_0 reading at the beginning of the analysis, the p_0 reading is repeated after the residual gases have been ejected from the chamber, and this final p_0 is used to calculated P_R. With changing temperature p_0 changes parallel with the vapor tension of moisture in the chamber, and therefore rises about 0.13 mm. with each 0.1° temperature increase in the range 20° to 25°.

Calculation

The pressure of oxygen P_{O_2}, in the sample at 50 cc. volume, is calculated as

$$P_{O_2} = 1/3\ (P_S + P_{H_2} - P_R)$$

$$\text{Per cent } O_2 \text{ in air} = 100 \times \frac{P_{O_2}}{P_S}$$

Temperature correction. If the temperature of the manometric chamber at the P_{H_2} or P_R reading differs from the temperature at the P_S measurement, P_{H_2} is corrected by the factor T_S/T_{H_2}, and P_R by the factor T_S/T_R, as described on p. 108.

COMBINATION OF THE PRECEDING METHODS FOR CO_2 AND O_2 IN RESPIRATORY AIR

The above methods can be applied to a single sample of respiratory air. The oxygen is first determined by combustion. The combustion chamber is then disconnected and the CO_2 in the residual gas is absorbed with alkali solution, and determined as described above on pages 119 to 122.

HYDROGEN BY COMBUSTION. VAN SLYKE AND HANKE (22)

The method is the same as the combustion method for oxygen previously described, except that here an excess of oxygen must be added in order to provide for the combustion of all the hydrogen. The same apparatus, shown in figure 13, is used. The only reagent required is oxygen, which in analyses of gas with small proportions of hydrogen can conveniently be added in the form of air. When the gas contains a large proportion of hydrogen, however, the use of air would involve an undesirably great dilution of the sample, and pure oxygen is used. Explosive mixtures are made to burn without explosion, as in the oxygen determination, by reducing their pressure below atmospheric.

Measurement of sample

The sample is admitted and measured as described above for oxygen determination by combustion.

$$P_S = p_1 \quad p_0.$$

After its measurement the sample is transferred to the combustion chamber, also as described in the oxygen analysis.

Addition of oxygen or air

With the sample in the combustion chamber, enough air or oxygen to burn the hydrogen is measured into the manometric chamber. The air or oxygen is admitted in the manner described on p. 111 for "admission of sample estimated by pressure."

Whether air or oxygen will be used, and how much of either, depends upon the hydrogen content of the sample. The pressure of oxygen present in the mixture burned must be at least half the pressure of the hydrogen. One can always measure an amount of oxygen into the chamber which will give more than 0.5 P_S mm. of pressure, and be sure that a sufficient excess is present, even if the sample is pure hydrogen.

If the sample contains less than 40 per cent of hydrogen, enough air to to give the same pressure at 50 cc. as the sample will provide sufficient oxygen for the combustion. However, in this case the P_S of the sample should not exceed 300 mm., or the amount of residual gas may be too great to measure in one portion. It is preferable, if the hydrogen content of the sample exceeds 10 per cent, to add pure oxygen rather than air for the combustion. One can then take always a full sized sample, with P_S of 500 mm.

The minimum amounts of air or oxygen that must be added, for samples of different H_2 and O_2 contents, are indicated by the following formulas. The formulas make allowance for any oxygen already in the sample. If P_{O_2} or P_{air} calculated by these formulas is zero or negative, enough oxygen is already present in the sample to burn the hydrogen, and addition of oxygen is unnecessary.

1) $$P_{O_2} = \left(\frac{\text{per cent } H_2}{200} - \frac{\text{per cent } O_2}{100}\right) \times P_S$$

2) $$P_{air} = \left(\frac{\text{per cent } H_2}{40} - \frac{\text{per cent } O_2}{20}\right) \times P_S$$

P_{O_2} = pressure of O_2 measured in chamber at 50-cc. volume; P_{air} = pressure of air similarly measured; per cent H_2 = per cent H_2 in gas sample; per cent O_2 = per cent O_2 in gas sample.

After the desired amount of air or oxygen has been admitted to the chamber, the mercury is brought to the 50 cc. mark, and reading p_2 is taken on the manometer.

$$P_A = p_2 - p_0.$$

P_A represents the pressure at 50 cc. of the added air or oxygen.

After p_2 has been noted the air or oxygen is run over into the combustion chamber in the same manner described for transfer of the sample.

Combustion

The manner of combustion depends upon the proportion of hydrogen in the gas mixture burned. If there is less than 10 per cent of hydrogen the mixture will burn quietly at atmospheric pressure without an explosion. If the hydrogen is between 10 and 20 per cent the mixture will burn at atmospheric pressure by explosion, the vigor of which increases with the proportion of hydrogen present. If the hydrogen is less than 20 per cent, however the explosion is not severe enough to break the glass combustion chamber. The concentration of excess oxygen in the mixture markes no appreciable difference in the vigor of the explosion. A mixture with 9 per cent of hydrogen will burn without explosion even if the rest of the gas is pure oxygen. And a mixture with 20 per cent of hydrogen explodes with no more apparent violence if the rest of the gas is pure oxygen than if it is 10 per cent oxygen.

Even the most explosive mixture, 2 parts of H_2 to 1 of pure O_2, will burn without explosion if the pressure is so reduced that the partial pressure of hydrogen is below 70 mm. Such reduction of the hydrogen pressure is obtained by reducing the total pressure of the mixture to 100 mm.

The per cent of hydrogen in the mixture to be burned is estimated as

$$\text{Per cent } H_2 \text{ in mixture burned} = (\text{Per cent } H_2 \text{ in sample}) \times \quad P_S + P_A$$

A. Combustion in one portion at atmospheric pressure. **If the proportion of hydrogen in the gas mixture burned is under 15 per cent, one may safely burn the mixture in one portion. After the sample and the added oxygen have been transferred to the combustion chamber its leveling bulb is put at such a height that the mercury in the chamber is a few millimeters above the floor of the chamber, and the wire is heated. If a flash occurs the combustion is complete at once. Otherwise the wire is kept at a red glow for 10 seconds. The current is then shut off, and fifteen to thirty seconds are allowed for the glass ends, into which the platinum wires are sealed, to cool so that contact with the mercury will not crack them. Then the leveling bulb of the combustion chamber is placed in the position shown in figure 13, and the cock at the top of the manometric chamber is turned to connect the two chambers. By opening the cock leading to the manometric leveling bulb the greater part of**

the gas is drawn over into the manometric chamber. The gas is then returned to the combustion chamber in the manner previously described in order to wash out any slight pockets or bubbles that may have escaped transfer to the combustion chamber the first time. With the leveling bulb of the combustion chamber as shown in figure 13 the wire is heated again for ten seconds to burn any traces of hydrogen that may have been washed in from the other chamber.

B. Combustion in instalments under diminished pressure. If the gas mixture contains more than 15 per cent of hydrogen it is burned under diminished pressure in order to prevent unduly violent explosions. In this case the mixture of gas sample plus air or oxygen, after being mixed in the combustion chamber, is passed back into the manometric chamber, and the cock between the two chambers is closed. If a large sample and much oxygen or air has been taken the total gas present may exceed 50 cc. at atmospheric pressure. In such a case 50 cc. is run back into the manometric chamber, and the rest is left in the combustion chamber. The sample should not be taken so large, however, that this excess is over 10 cc. The leveling bulb of the combustion chamber is lowered to a point 660 mm. below the floor of that chamber. The leveling bulb of the manometric chamber is left at the medium position, and the cock connecting the bulb and chamber is left open. Then the cock at the top of the manometric chamber is opened until enough gas has entered the combustion chamber to cause the mercury to fall to within a few millimeters of the floor of that chamber.

The cock connecting the two chambers is then closed, and the platinum wire is heated for ten seconds. As the gas burns and contracts the mercury is the combustion chamber rises somewhat. After the current is turned off, thirty seconds or more is allowed for the platinum wire to cool. Then about 10 cc. more gas are admitted from the manometric chamber, the leveling bulb of the combustion chamber being raised a little if necessary to keep the mercury in the chamber above the floor of the latter. The combustion is then repeated as before. This process is repeated until all the gas has been transferred to the combustion chamber and submitted to combustion. The thirty seconds wait after each combustion before the cock connecting the two chambers is reopened is ***never to be neglected,*** because if the cock is opened while the wire is still warm, even though all glow has ceased, the stream of entering gas striking the wire may ignite and the flash may strike back into the manometric chamber and produce an undesirable explosion.

After the last combustion the gas is once run over into the manometric

chamber and back to the combustion chamber, as in Procedure ***A***, and is exposed to the heated wire again. During this ignition the leveling bulb of the combustion chamber is raised 10 or 20 cm. above the chamber, so that the gas is under more than atmospheric pressure. The high pressure adds assurance of complete combustion of final traces of hydrogen.

If the gas mixture contains between 15 and 30 per cent hydrogen it may be burned in only two portions, the first under 1/2 atmosphere pressure, the second under pressure only a little less than atmospheric.

During the combustion in instalments by the above technique the capillary joint connecting the two chambers is not filled with mercury, but with the gas. It is possible that if the ignition occurred by explosion some gas might be lost by being pressed out through the rubber joint. It is therefore desirable to conduct these ignitions under sufficiently reduced pressure to obtain always quiet combustion, or at most only mild explosions.

Measurement of residual unburned gas

After the last heating of the wire, and the thirty second interval to permit the glass ends to cool, the unburned gas is returned to the manometric chamber, followed by a little mercury. The mercury meniscus is lowered to the 50-cc. mark, and allowed to rest there two minutes while the gas cools. Then p_3 is read.

The pressure, P_R of the residual unburned gas is calculated as

$$P_R = p_3 - p_0$$

In case the temperature in the water jacket has changed since the p_o reading was made when the sample was measured, the p_o reading is repeated after the residual gas has been ejected, from the chamber. The p_o now obtained is used to calculate P_R by the above formula. The p_o changes with vapor tension, as stated in discussing this stage of the oxygen determination by combustion.

If the temperature at the P_A or P_R reading differs from the temperature at the P_S measurement, P_A or P_R, or both, are corrected as described on p. 108.

Calculation

$$P_{H_2} = 2/3\ (P_S + P_A - P_R)$$

$$\text{Per cent hydrogen} = 100\ P_{H_2}$$

GASES OTHER THAN HYDROGEN BY COMBUSTION

The conditions for the determination of carbon monoxide, methane, and other gases by combustion combined with measurement of the CO_2 formed have been mentioned on page 102 in connection with Haldane analyses. The same principles can doubtless be utilized with the manometric apparatus, the CO_2 analyses being performed on the burned gases by the methods described in the preceding pages.

HYDROGEN BY ABSORPTION WITH A SOLUTION OF COLLOIDAL PALLADIUM AND SODIUM PICRATE. VAN SLYKE AND HANKE (23)

The reagent used is a solution of colloidal palladium and sodium picrate introduced by Paal and Hartmann (16). The palladium catalyzes the reduction of the picrate by absorbed hydrogen gas. The entire analysis is carried out in the manometric chamber, and is especially adapted to analyses of small samples of gas. The limit of error is about 0.2 volume per cent. It is therefore less accurate than the macro analysis by combustion described above. It is, however, convenient for micro analyses, and enables one to perform a hydrogen determination when a combustion chamber is not at hand. *Carbon dioxide and oxygen must both be removed before the hyrogen is determined.* The CO_2 would be absorbed by the alkaline picrate solution, and the oxygen would interfere with the absorption of hydrogen.

Reagents

Palladium sodium picrate solution. This solution contains 2 per cent colloidal palladium ("nach Paal") (16) and 3.5 per cent picric acid in 0.154 N NaOH. To 3.5 g. picric acid, 15.4 cc. N NaOH and about 50 cc. of water are added, and the mixture is warmed to about 50° until the picric acid is dissolved. Then 2 grams of colloidal palladium, previously stirred up with about 20 cc. of water, are added, and all is made up to 100 cc. It takes about one hour with occasional stirring for the solution to become entirely homogeneous.

Since colloidal palladium is gradually inactivated by contact with metallic mercury, it is necessary to avoid introducing mercury into the reagent accidentally. The stock reagent should be kept in a stoppered bottle at a distance from the manometric apparatus in order to avoid contamination with the mercury droplets that are likely to pervade the surroundings. The portion of reagent used is kept in a small flask containing only 5 or 10 cc.

Paal (16) used a palladium solution containing 5 per cent picric acid, instead of the 3.5 per cent here described. From the solution with 5 per cent picric acid a considerable amount of sodium picrate gradually precipitates on standing. By decreasing the concen-

tration to 3.5 per cent, this difficulty can be entirely avoided. Assuming a reduction of the picric acid to tri-amino-phenol by the hydrogen, 0.2 cc. of the reagent is equivalent to 5.5 cc. of hydrogen at one atmosphere. Therefore the method as described provides a 4-fold excess of reagent, even when pure hydrogen is analysed.

The colloidal palladium used by Van Slyke and Hanke was purchased from Dr. Theodor Schuchardt, Chemische Fabrik, Görlitz, Germany, for $8.25 a gram. Using 0.2 cc. of a 2 per cent solution, or 4 mg., makes the palladium cost about 3 cents for each analysis.

Gas-free water stored in a Hempel pipette over mercury. Distilled water is deaerated by the procedure described on page 235 for the preparation of gas-free reagents. It is then stored in a Hempel pipette over mercury as shown in figure 12. In the present cause the the fluid shown in figure 12 is mercury, and the gas is replaced by air-free water.

Measurement of gas sample

The gas sample is conveniently stored 'n a modified Hempel pipette as shown in figure 11. As displacement liquid in the pipette one may use alkaline pyrogallol or hyposulfite solution, which absorb oxygen and carbon dioxide from the gas mixture and thereby prepare it for the hydrogen determination. If either of these solutions is used in the pipette of figure 11 the part of the solution in the upper bulb is covered with a layer of paraffin oil.

The p_0 reading is taken, with the mercury meniscus at the 2 cc. mark and the manometric chamber free of gas and fluid, as described on page 107.

A sample of about 1.5 cc. of the CO_2 and O_2-free gas is introduced into the manometric chamber as described for "admission of sample estimated by volume" on page 108, and the p_1 reading is taken with the mercury again at the 2 cc. mark. The pressure, P_S, exerted by the gas sample at 2 cc. volume is calculated as:

$$P_S = c_1 (p_1 - p_0)$$

The correction factor, c_1, which has a value of about 1.01, is discussed below.

Absorption of hydrogen with picrate palladium solution

The excess of mercury is removed from the cup, so that there is just a little more than necessary to fill the capillary. The leveling bulb of the apparatus is left in medium position, opposite the bottom of the chamber, and the cock between bulb and chamber is left open. 0.2 cc. of the palladium-sodium picrate reagent, measured with a pipette to within

0.01 cc., is introduced into the cup. With a copper wire any bubble of air between the palladium solution and the mercury is removed. Such a bubble can be avoided by having a little more mercury in the cup than is necessary to fill the capillary before allowing the reagent to flow into the cup. The reagent is now admitted into the chamber in 5 portions of approximately 0.04 cc. each. After the admission of each portion the mercury in the chamber is lowered and raised twice in the following manner, in order to expose the gas to a large surface of the palladium solution.

After the admission of a drop of palladium solution into the chamber the mercury is lowered to a level 2 or 3 cm. below the 2 cc. mark, and the cock leading to the mercury leveling bulb is closed. The sudden fall of the mercury leaves the palladium solution distributed more or less uniformly over the glass wall of the upper part of the chamber, and as the solution gradually drains down after the mercury a large surface of the solution is exposed to the gas. The mercury leveling bulb is placed in the medium position, level with the bottom of the chamber. After twenty or thirty seconds, when most of the palladium solution has drained down to the surface of the mercury in the chamber, the cock leading to the leveling bulb is opened slightly, allowing the mercury in the chamber to rise slowly, pushing the palladium solution ahead of it as far as it will rise.[7] The mercury is then lowered again 2 or 3 cm. below the 2-cc. mark, and the entire process is repeated. Then the next drop of palladium solution is admitted. The process is performed at such a rate that ten minutes are taken for admission of the 0.2 cc. of palladium solution and the absorption of the hydrogen. The cock of the chamber is sealed with mercury after the last instalment of palladium solution has been added.

During this manipulation it is important to avoid raising the leveling bulb so high that the gas in the chamber is under greater than atmospheric pressure. The rate of absorption of hydrogen by the palladium solution can be shown to increase as the total gas pressure increases, and such increase in gas pressure would be desirable if permissible. It must, however, be avoided since it may cause appreciable leakage of gas, even though the stopcock be well greased. Conversely it is important not to lower the mercury in the chamber

[7] If the mercury were allowed to rise rapidly most of the palladium solution would remain adherent to the glass walls of the chamber. Consequently a large surface of the solution would be exposed to the mercury, and the palladium would be rapidly inactivated. If the mercury rises gradually, in the course of about ten seconds, practically all of the palladium solution drains upward ahead of the mercury, thus making minimum contact with the mercury surface.

unduly, so as to decrease the gas pressure greatly, since the absorption of hydrogen is retarded at low pressures. For these reasons it is essential for the manipulation during hydrogen absorption that the mercury should be lowered each time only 2 or 3 cm. below the 2 cc. mark, and then allowed to rise as far as it will go with the leveling bulb in the medium position. This procedure keeps the gas under somewhat less than atmospheric pressure.

A smoothly turning, well greased cock is needed to control the addition of as little as 0.04 cc. of solution into the reaction chamber. To assist still further in controlling the flow of liquid from the cup to the chamber, the pressure of the gas in the chamber can be adjusted to be just slightly less than atmospheric by holding the leveling bulb so that its mercury level is about 1 or 2 cm. lower than the level of the mercury in the chamber, and then closing the stop-cock between the leveling bulb and the chamber. Even if the stop-cock above the chamber is now opened wide, only a little liquid will enter the chamber. The volume of liquid admitted will in fact be exactly 0.04 cc. if the volume of the gas is 1.5 cc. and the difference in mercury levels is 2 cm. The practiced analyst will find it more convenient to leave the leveling bulb and chamber connected and to depend entirely upon the well-greased stop-cock of the reaction chamber for controlling the addition of reagent, even for the last instalment; but this method requires a degree of control over the stop-cock which comes only with experience.

Addition of air-free water and measurement of the residual gas

After the hydrogen has been absorbed 2 to 2.5 cc. of gas-free water are admitted to the chamber. The water dilutes the palladium solution so that it drains more readily, and so that it becomes transparent and its meniscus thus more accurately definable.[8]

To admit the air-free water the Hempel pipette containing it is placed as shown in figure 11, the outlet capillary of the pipette having been first filled with mercury from the cup above. While admitting the water it is convenient to hold the outlet capillary of the Hempel pipette firmly in the cup with the left hand, and to use the right to turn the stopcocks. It is not necessary to measure the water added more accurately than to keep its volume between 2.0 and 2.5 cc. The measurement is easily made by estimating the water volume from the volume of residual gas in the chamber and the position of the mercury meniscus with respect to the 2-cc. mark. If the volume of water is not thus approximately controlled, a slight but measurable error may be introduced because of different quantities of the residual gas later dissolved by the aqueous phase.

[8] It is not sufficient here to use gas-free water which is stored under oil, nor is it permissible to run the water into the cup, and from there into the chamber. Such manipulation would permit absorption of enough air into the water to affect the results. It is necessary to store the water over mercury and to deliver it from its container directly through a mercury seal into the reaction chamber without contact with air.

After the water is admitted the Hempel pipette is removed and the stop-cock is sealed with mercury. The mercury meniscus is then lowered to the 50-cc. mark and the chamber is shaken for two minutes. Thereby slight amounts of gas which may have been absorbed by the water during its admission are returned to the gas phase; only about 0.001 of the total N_2 or H_2 in the chamber remains dissolved under the conditions of equilibration. The water meniscus is then brought to the 2-cc. mark and the manometer reading p_2 is taken. The temperature is recorded.

It remains to take the zero reading in the absence of gas with the diluted palladium solution present. The gases are ejected without loss of liquid as described on page 279. The stop-cock is sealed with mercury, the water meniscus is brought exactly to the 2-cc. mark and the manometer reading, p_3 is taken. Expressing the pressure of the residual gas as P_R we have

$$P_R = p_2 - p_3 - c_2$$

After the p_3 reading the used reagent is ejected and the reaction chamber washed twice with water.

Determination of the corrections c_1 and c_2

The correction factor c_1 for the difference between mercury and water menisci. Because the water meniscus curves upwards above the 2-cc. mark while the mercury meniscus curves downwards from it, the volume of gas measured at the 2-cc. mark above water is about 1 per cent less than that measured at the same mark above mercury. The pressure of a given amount of gas measured above the water meniscus is accordingly about 1 per cent more than that of the same amount of gas measured above the mercury meniscus. Consequently in order to calculate what the P_S pressures would be if measured over water, as is the P_R pressure, it is necessary to multiply the observed $p_1 - p_0$ pressures by a factor, c_1, which has a value of about 1.01. The exact value of this factor depends on the diameter of the chamber of the 2-cc. mark. It is determined empirically by measuring the pressures exerted by any convenient amount of gas brought to the 2-cc. mark first over mercury and then over water.

The correction is determined as follows. A reading, p_0 is taken with no gas or visible water in the chamber, and with the mercury meniscus at the 2-cc. mark. Then enough air is admitted to exert 400 to 500 mm. pressure at 2-cc. volume, and p_1 reading is taken, again over mercury.

Two cubic centimeters of air-free water are now admitted to the chamber, the mercury is lowered to the 50-cc. mark, and the chamber is shaken for two minutes. The water meniscus is now brought to the 2-cc. mark and the reading p_2 is taken. Finally the air is ejected from the chamber, without loss of more than 0.1 cc. of the water, and the reading p_3 is taken with the chamber gas-free and the water meniscus again at the 2-cc. mark. Then

$$c_1 = \frac{p_2 - p_3}{p_1 - p_0} \quad \frac{\text{pressure over water}}{\text{pressure over mercury}} = \text{about } 1.01.$$

The exact value of c_1 is determined by repeating several times the above determination and taking the average result. The value of c_1 can be thus determined with a precision of about 1 part per 1000.[9]

The correction factor c_2 for air carried into the chamber dissolved in the 0.2 cc. of palladium solution. The palladium solution when admitted is saturated with air, which it gives off in the chamber. To determine c_2 1 cc. of the palladium solution and 1 cc. of air-free water are measured into the chamber. The mercury is lowered to the 50 cc. mark and the solution is extracted by shaking for 2 minutes. The pressure p_1 is then measured at 2 cc. volume. The gas is ejected, and p_3 is read.

$$c_2 = 0.2\ (p_1 - p_2)$$

In this case five times the usual amount of palladium solution is taken, for greater, accuracy and the pressure of the extracted air is divided by 5 to obtain the correction for the amount of reagent used in the analyses. The c_2 correction is usually about 1.5 mm.

Correction for temperature changes. If the temperature at the P_R measurement differs significantly from that at the P_S measurement, the observed P_R is corrected as described on p. 108.

[9] The c_1 value also includes a slight correction for residual gas (usually N_2) which remains dissolved in the 2 cc. of water when the latter is extracted in the evacuated chamber and then raised to the 2-cc. mark. The amount remaining in the extracted water, calculated from the solubility of N_2, is approximately 0.1 per cent of the total N_2 in the system. However, the amount determined in control experiments has been found to be 0.2 per cent. The extra 0.1 per cent is attributable to reabsorption of N_2 by the water solution as it rises, after the extraction, from the bottom of the chamber to the 2-cc. mark. The correction for the 0.2 per cent is automatically included in the c_1 correction when the latter is determined as above outlined.

Calculation

A. Hydrogen in CO_2- and O_2-Free Gas analyzed

$$P_{H_2} = P_S - P_R$$

$$\text{Per cent } H_2 = 100 \times \frac{P_{H_2}}{P_S}$$

B. Hydrogen in Original Gas Containing CO_2 and O_2

$$\text{Per cent } H_2 = A \times \frac{100 - (CO_2 + O_2)}{100}$$

A = per cent H_2 calculated by formula A; $(CO_2 + O_2)$ = per cent of $CO_2 + O_2$ in original gas mixture.

CARBON MONOXIDE

A. GAS MIXTURES CONTAINING LARGE AMOUNTS OF CARBON MONOXIDE

If the gas contains 10 per cent or more of carbon monoxide it can be analyzed by the technique developed by Sendroy and Liu (20) for analysis of the gases from blood containing CO. The CO_2 and O_2 are first absorbed with alkaline pyrogallate solution, and the CO in the residual gas is absorbed with cuprous chloride solution, as described on p. 335.

Or the CO may be burned to CO_2 by means of the technique described above for hydrogen determination by combustion, the CO_2 then being measured as described in the foregoing parts of this chapter. Minute amounts of CO in air can not well be determined by combustion, because other carbon containing gases or particles are likely to be present in sufficient amount to interfere (Sendroy, unpublished results).

B. GAS MIXTURES CONTAINING SMALL AMOUNTS OF CARBON MONOXIDE. SENDROY (19)

For mixtures containing small amounts of carbon monoxide a principle employed by Arnold, Carrier, Smith and Whipple (1) and others is utilized. The gas sample is freed of oxygen, after which the carbon monoxide is absorbed by reduced blood. The CO is then determined by the usual technique for determining carbon monoxide in blood. In the absence of oxygen the affinity of reduced blood for carbon monoxide is so great that even when the concentration of CO in the gas is only 0.05 per cent it is absorbed by the blood to the extent of 94 per cent.

With the manometric apparatus and refinements in the technique, Sendroy (19) is able to determine 0.05 to 0.3 volume per cent of CO in air

with a maximum error of ±5 per cent of the amount determined. The present method is well suited for quantitative determination of the amounts of carbon monoxide in air that are of physiological significance. The carbon monoxide estimation can conveniently be combined with an oxygen determination on the same air sample by the method described on page 112.

TABLE 16

FACTORS BY WHICH P_{CO} IS MULTIPLIED TO GIVE 100 TIMES THE CUBIC CENTIMETERS OF CARBON MONOXIDE PRESENT IN THE GAS SAMPLE ANALYZED*

TEMPERATURE	FACTOR f_1 WHEN IS P_{CO} MEASURED WITH GAS AT 0.5-CC. VOLUME	FACTOR f_1 WHEN P_{CO} IS MEASURED WITH GAS AT 2-CC. VOLUME
°C.		
15	0.0674	0.2696
16	71	84
17	69	73
18	67	63
19	64	54
20	62	46
21	60	38
22	57	26
23	55	17
24	52	08
25	49	0.2598
26	47	88
27	45	78
28	43	68
29	40	59
30	38	50
31	35	41
32	33	32
33	31	23
34	29	14

* These factors are obtained by multiplying by 5 × 1.067 those in table 32 on page 331. The factors in table 32 give 20 × cc. CO present in sample. Hence multiplying by 5 gives 100 × cc. CO present. The factor 1.067 is a correction for the fact that only 93.7 per cent of the CO in the air sample is absorbed by the blood.

Reagents

With the following exceptions, the reagents are those required for the method of Sendroy and Liu described on page 331, chapter VII.

(a) The air-free 1 *N* NaOH is not required.

(b) *Sodium hyposulfite solution without catalyst,* for blood reduction, is also prepared as described on page 113 and is kept for the day's work under oil.

(c) *Fresh ox blood.* This is reduced as described below.

Measurement and deoxygenation of gas sample

The cleaned chamber of the manometric apparatus is connected through the capillary side arm at the top with a gas sample container of about 40 or 50 cc. capacity, with leveling bulb attached, as shown in figure 12. The container is filled with mercury, as are the connecting tubes, so that no gas remains in the system. For the connection a rubber tube with heavy walls and small bore is used; the tube should be just long enough to permit shaking of the chamber without inconvenient tugging on the gas container.

From another container, the sample of gas, about 35 cc., is run into the chamber, P_S is measured as p_1-p_0, and the oxygen is absorbed, as described on p. 113–114. In this analysis, however, after deoxygenation the unabsorbed gas is not ejected through the stop-cock of the chamber, into the room, but is run through the side arm to the attached container. The hyposulfite is permitted to follow the gas up into the narrow part of the chamber until the solution ***just*** touches the stop-cock. The stop-cock is then turned, the hyposulfite is ejected into the cup above the chamber, and the chamber is cleaned with water. The deoxygenated gas sample is kept in the attached container under atmospheric pressure, until ready for equilibration with the reduced blood.

Determination of oxygen

In the foregoing procedure, if the oxygen content of the gas sample is desired, it may easily be obtained by reading p_2, as described on p. 114 before the deoxygenated gas is removed from the chamber. (The CO_2, is absorbed with the oxygen, but in samples of atmospheric air the CO_2 content is relatively negligible.) After the O_2-free gas is run into the attached container, a little hyposulfite is run into the capillary leading to the cup of the chamber, the stop-cock is sealed with mercury, and the reading p_3 is taken. The hyposulfite is then ejected and the chamber is washed with water. For calculation, see page 151.

Removal of air from ferricyanide reagent

One drop of caprylic alcohol and 15 cc. of the acid ferricyanide reagent are run into the chamber of the manometric apparatus and freed of air by

extraction for two three-minute periods, as described on page 322, chapter VII. The gas-free reagent is then forced up into a 25-cc. burette in the manner shown in figure on page 234. The burette should contain sufficient oil to make a layer at least 10 cm. deep, and the tip, which must extend long enough to reach the bottom of the chamber cup, is provided with a rubber ring to make a tight connection. The burette is set aside until the reduced blood has been prepared for analysis.

Preparation of reduced blood

A 5-cc. portion of ox blood of normal hemoglobin content is run into the cleaned chamber of the manometric apparatus, 2 drops of caprylic alcohol are added, and the chamber is evacuated and shaken for three minutes. The extracted gases are ejected without loss of solution as described on page 279. One drop of the sodium hyposulfite solution without catalyst is added to the blood and the extraction *in vacuo* is then repeated, this time for two minutes. After this treatment, there is no measurable amount of oxygen left in the blood. After each extraction the ejection of the gas from the chamber must be complete. If a drop of blood follows the ejected bubble of gas up into the cup its loss is of no importance. Since the drop is exposed to air in the cup the blood is not returned to the chamber, but is washed out of the cup before the cock is sealed with mercury for the next extraction.

Absorption of carbon monoxide by reduced blood

The deoxygenated gas from the attached container is admitted into the chamber over the reduced blood. It is followed by a little mercury, to fill the connecting tubes, and to seal the stop-cock of the chamber. After this the attached container is disconnected.

The water jacket of the monometric chamber is completely covered with tin foil to exclude all light; the tin foil may be fastened with adhesive tape. With the leveling bulb of the chamber in a position slightly lower than the bottom of the chamber, and with the cock between the two open, the chamber is slowly shaken for thirty minutes. The speed of shaking should be such that the blood is whirled about the wall but is not broken up into foam. After equilibration the tin foil is removed and the unabsorbed gas is ejected without loss of any of the blood. Three drops of caprylic alcohol are added to the reduced blood in the chamber, and the cock of the chamber is sealed with mercury.

Determination of CO absorbed by the blood

Through a mercury seal (fig. 52, p. 344) 13 cc. of the ferricyanide reagent from the oil-protected burette are admitted into the chamber with the blood. Before the acid ferricyanide is admitted the chamber is partly evacuated, so that the surface of the blood is in the broad part of the chamber. After each 2 cc. admitted, the chamber is shaken gently two or three times to avoid the formation of large masses of protein precipitate which would be likely to adhere to the glass walls.

From this point the procedure for determination of CO in the blood is precisely that described for 5 cc.-blood samples on page 332 for the Sendroy-Liu method, except that in the present case no alkali need be introduced into the chamber before the liberated gases are run into the Hempel pipette containing alkaline pyrogallate. The measurements p_4 and p_5, before and after the absorption of the CO with Winkler's solution, are made with the gases in the chamber at 0.5-cc. volume.

Calculation

The calculation for O_2 is similar to, but somewhat simpler than that given on page 115.

As before, the sample is calculated as

$$P_S = p_1 - p_0.$$

$$P_{N_2 + CO} = 0.94\ (p_2 - p_3)$$

The oxygen, (including a slight amount of CO_2 which is negligible in ordinary atmospheric air) is calculated as

$$P_{O_2} = P_S - P_{N_2 + CO}$$

$$\text{Per cent } O_2 = \frac{100\ P_{O_2}}{}$$

The CO is calculated as

$$P_{CO} = p_4 - p_5 - c$$

$$\text{Per cent CO } \frac{f_1\ P_{CO}}{f_2\ P_S}$$

f_1 = factor from table 16 by which P_{CO} is multiplied to give 100 × the cubic centimeters of CO, reduced to 0°, 760 mm., present in the gas sample.

f_2 = factor from table 15A by which the pressure difference P_s is converted to the volume of the sample in cubic centimeters reduced to 0°, 760 mm. (see p. 130).

The c correction is determined by repeating the procedure described above in all respects, except that the steps involved in handling the air sample are omitted. The reagent is deaerated, the blood is reduced, the reagent is added, and the determination finished as above. Two readings are made at the 0.5-cc. mark, p_1 and p_2, before and after the addition of Winkler's solution to absorb CO. The c correction is calculated as: $p_1 - p_2 = c$.

The c correction is the sum of two components. One is the c correction of the Sendroy-Liu method. The other is a correction for a slight amount of CO (0.08 to 0.18 volume per cent) which Sendroy, in agreement with Nicloux and others, has found to be present in normal blood. Because this CO content of the blood is variable, the c correction must be determined with a portion of the same blood which is used for the air analysis.

The correction factor 1.067

Since the reaction, $Hb + CO \rightleftarrows HbCO$, is reversible, it is theoretically impossible that, when the $N_2 + CO$ mixture is shaken with blood, absolutely all of the CO should be combined. Under the conditions here used for the absorption of CO by reduced blood, Sendroy has found that only 93.7 ± 2.4 per cent of known amounts of CO (in the small concentrations determined) in air is recovered from the reduced blood used for the analysis. The correction factor 1.067 has been found constant to within ±2.4 per cent, and is accordingly used in the calculation.

Gas Analyses with Special Apparatus*

CARBON DIOXIDE IN AIR BY THE COLORIMETRIC METHOD OF HIGGINS AND MARRIOTT (10, 13)

Principle

The air sample is bubbled through a standard bicarbonate solution containing a measured amount of phenol red as indicator until the H_2CO_3 content of the bicarbonate solution is at equilibrium with the CO_2 tension in the passing air. The color of the solution is then compared in a comparator with a series of buffer solutions of known pH. From the pH, thus deter-

* See the appendix for a method by Y. Henderson which appeared too late for inclusion in this chapter.

mined, the CO_2 content or tension of the air can be estimated, since in accordance with Hasselbalch's equation (see p. 874–883 of volume I) the CO_2 tension can be calculated if bicarbonate content and pH are known. In practice one uses as standards a set of standard tubes which indicate CO_2 tensions directly, so that no calculations are necessary.

The method is of somewhat limited accuracy. However, it affords a simple means of securing approximate information concerning the CO_2 tension in the alveolar air when results accurate to 2 or 3 mm. of CO_2 tension suffice. The method has been used by Marriott (13) extensively in determining the CO_2 content of alveolar air obtained by the Plesch method from infants. The simplicity of the technique makes it available as a bedside method.

Reagents

Phosphate buffer mixtures. M/15 solutions of alkaline and acid phosphate are made as follows. For the acid phosphate, 9.08 grams of anhydrous KH_2PO_4 are dissolved in 200 cc. of 0.01 per cent phenol red solution and made up to 1 liter. For the alkaline phosphate, 9.47 grams of anhydrous Na_2HPO_4 or 11.88 grams of $Na_2HPO_4 \cdot 2H_2O$ are dissolved in 200 cc. of 0.01 per cent phenol red and made up to 1 liter. The acid and alkali phosphate solutions are mixed in varying proportions, as indicated by table 17.

Standard 0.01 N *alkali.* To 100 cc. of 0.1 N sodium hydroxide in a 1 liter volumetric flask, add 200 cc. of 0.01 per cent phenol red. Dilute the solution to a liter. The hydroxide can be converted to bicarbonate by passing through it carbon dioxide from a cylinder. However, this is unnecessary, as change from NaOH to $NaHCO_3$ occurs automatically during the determination.

Procedure

A series of tubes 10 by 100 to 150 mm., containing acid and alkaline phosphate solutions in the proportions indicated in table 17, is prepared. The tubes are sealed or the solutions are covered with low melting point paraffin. The tubes should be kept in the dark when not in use. They must be renewed, as the colors fade, and should be checked frequently.[10] The CO_2 tension equivalent of each tube is indicated in table 17.

Into a similar tube are poured 2 to 3 cc. of standard 0.01 N alkali solution. Through this the air is bubbled, by means of a glass tube drawn

[10] Comparator and standard buffer solutions in sealed tubes are made by Hynson, Westcott and Dunning of Baltimore.

out to a fine capillary point, until the color of the solution becomes constant. The test tube is then stoppered, or the solution is covered with oil, and the color of the solution is compared with the standards in a comparator. The tension of CO_2 is read directly on the standard tube which most nearly matches the unknown. The bicarbonate solution must be at 20° to 25° while the gas is being passed through it. If the temperature of the room is outside these limits the tubes should be immersed in water at about 25°. No correction for barometric pressure is required.

TABLE 17

TABLE FOR USE WITH HIGGINS AND MARRIOTT'S METHOD FOR ALVEOLAR CO_2 DETERMINATION

	CO_2 TENSION, *mm. Hg*							
	10	15	20	25	30	35	40	45
Acid phosphate, *cc*.	17.8	25.2	31.0	35.7	40.5	45.0	47.0	50.2
Alkaline phosphate, *cc*.	82.2	74.8	69.0	64.3	59.5	55.0	53.0	49.8

CARBON DIOXIDE AND OXYGEN DETERMINATION BY ELECTRICAL MEASUREMENT OF THERMAL CONDUCTIVITY OF AIR

Hill (9) in 1922 showed that the effect exerted by CO_2 on the thermal conductivity of air could be used for rapid electrometric determination of the CO_2 content. Rabinowitch and Bazin (17) confirmed Hill and described "kathetometers" adapted for the determination. Finally Ledig and Lyman (12) utilized the principle for determination of both CO_2 and O_2. They could complete an analysis in ten minutes, with results as accurate as those yielded by the Haldane method.

There is no doubt that these methods are rapid and exact. Because they require unusual technique and apparatus, however, they have apparently not been used outside the laboratories of their originators. The reader will find descriptions in the papers above cited.

THE DETERMINATION OF SMALL AMOUNTS OF CARBON MONOXIDE IN AIR BY THE IODINE PENTOXIDE METHOD. SENDROY'S MODIFICATION OF VANDAVEER-GREGG TECHNIQUE (21)[11]

This procedure is more time consuming and difficult than the manometric method of Sendroy described on page 147. It serves, however, when the

[11] For the following description, which includes details not yet published, the authors are indebted to Dr. J. Sendroy, Jr., of the Rockefeller Institute.

manometric apparatus is not available, or when results accurate to 1 or 2 per cent of the CO present are desired.

The method utilizes the reaction of CO with hot I_2O_5.

$$5\ CO + I_2O_5 = 5\ CO_2 + I_2$$

The liberated iodine is absorbed in KI solution and titrated against sodium thiosulfate in the presence of starch indicator. When the apparatus is properly assembled and the conditions of analysis are rigidly observed, many analyses may be made without difficulty or change in the assembly. Results for concentrations of CO in air from 0.01 to 0.2 volume present are accurate to ± 1.5 per cent of the CO determined.

Apparatus

The assembly is shown in figure 14. Gas from reservoir A or B (capacity about 800 cc.) is displaced by glycerol-salt solution and is passed through the trap T (volume about 30 cc.) where any overflow of glycerol-salt solution through stop-cock B is caught. The gas is purified by being passed in turn through the sealed U-tube S (5-inch height) containing glass beads moistened with concentrated sulfuric acid, and through the glass-stoppered U-tube P (5-inch height) containing soda-lime in one arm separated by glass wool from P_2O_5 in the other. Next, the gas enters the sealed U-tube X (6-inch height) containing alternate layers of glass wool and Merck's I_2O_5 (iodic acid anhydride). Tube X is kept in an electrically heated oven at a constant temperature, so that during analysis the air within is at 150°C. The free iodine is absorbed in tube K (volume about 50 cc.) containing KI solution. As a further precaution, tube K' is added with additional KI solution to absorb any iodine which may escape from tube K.

Reagents

Glycerol-salt solution. One volume of glycerol is mixed with three volumes of saturated NaCl solution. It is used rather than water in A and B because it dissolves less gas.

Potassium iodide solution. This should be prepared fresh daily. One to 10 grams of KI, depending on the concentration of CO in the gas sample, are dissolved in 100 cc. of distilled water. The purity of the KI should be tested: any lot which liberates free iodine in solution within less than twenty four hours when kept in the dark should be rejected.

Sodium thiosulfate solutions. A 0.1 N stock solution is prepared as described in chapter I. Dilute solutions varying in strength from 0.01 to

0.001 N are made at frequent intervals from the above. These dilute solutions are unstable, and must be standardized on the day they are used, by titration against similarly dilute biiodate solutions.

Potassium biiodate solutions. A 0.1 N stock solution is prepared as described in chapter I, and from it 0.01 N and 0.001 N are prepared by dilution.

Starch indicator solution. Described in chapter I.

Procedure

The iodine pentoxide is prepared for use by heating in tube X at a temperature of 210°C. for about two days while nitrogen from the N_2 tank is drawn through the system at the rate used in analysis. The temperature is then dropped to 150°C. and the purging with nitrogen is continued for about two days more. After this, the I_2O_5 should liberate no trace of iodine when blank analyses are run with nitrogen.

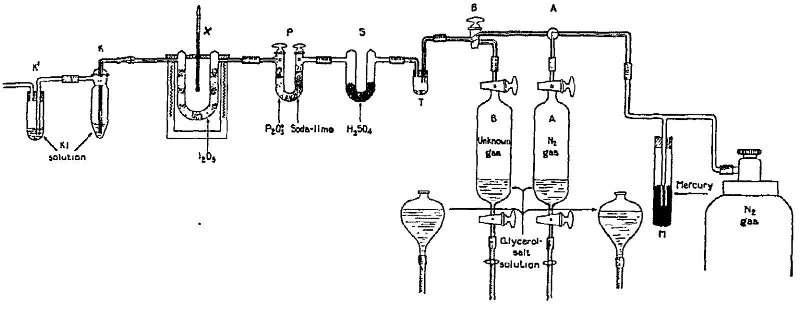


FIG. 14. Apparatus for determination of carbon monoxide in air by iodine pentoxide method.

Each day's analysis is begun by doing a blank on nitrogen (commercial). The gas obtained from the N_2 cylinder (fig. 14) is run through the mercury trap M, through stop-cock A, into the reservoir A. After the I_2O_5 tube has been heated at 150°C. for one-half hour with nitrogen running through continuously, the tubes K' and K, each containing 5 cc. of solution, are placed in position. By controlling the inflow of glycerol-salt solution into A, the outflow of N_2 gas is regulated at such a rate that the 800 cc. of N_2 are displaced from A in one and one-half to three hours. The tubes K and K' are detached. Titration should indicate no more iodine than will oxidize 0.01 or 0.02 cc. of the thiosulfate.

Before the calibrated tube B is attached it is completely filled with a sample of the air to be analyzed. It is then immersed in a water bath

at a temperature a little higher than room temperature. When thermal equilibrium has been attained the top stop-cock is opened to the atmosphere for some seconds to let warmed air escape until the pressure in *B* has fallen to atmospheric. *B* is then closed, and the temperature of the bath and the prevailing barometric pressure are recorded. The gas sample is then ready for analysis.

Tubes *K* and *K'* are placed in position. When the concentration of CO in the air analyzed is about 0.01 per cent, a 1 per cent solution of KI is used in tubes *K* and *K'*. When 0.1 per cent of CO or more is expected, 5 per cent KI solution is used.

The gas to be analyzed is now run through the system at the same rate previously used for the nitrogen. During this time tube *A* is refilled with nitrogen. After tube *B* has been completely emptied, (with a trace of glycerol-salt solution through stop-cock *B*), the N_2 gas in tonometer *A* is used to wash out the system. Then the KI solution is titrated against thiosulfate.

Titration. After quantitative rinsing of the inlet of tube *K* with 1 cc. of water, the tube, with its stopper, is disconnected from the train, and the contents are titrated against thiosulfate solution delivered from a Bang 3 cc. microburette of the type described in chapter I. When enough thiosulfate has been added to discharge the yellow color in *K*, two drops of starch solution are added. The titration is completed to the blue-to-white end-point. The solution in tube *K'* is similarly titrated. For analysis of air with less than 0.01 per cent of CO thiosulfate solution of 0.001 N concentration is used. When samples of air con ain 0.1 per cent CO or more the titration should be made with 0.01 N thiosulfate.

In standardizing the thiosulfate, a 50 cc. tube similar in size and shape to tube *K* is used. Two cubic centimeters of the appropriate biiodate (dilute solutions from the stock solution should be made fresh about every two weeks) are accurately pipetted into the tube, 4 cc. of the strength KI used are added, and then sufficient HCl (2 drops of 1 N HCl when the biiodate is 0.01 N) is added to complete the reaction. Titration to the end-point is completed as described above.

Calculation

From the reactions involved, 1 cc. of 0.001 N $Na_2S_2O_3$ is equivalent to 0.056 cc. of CO measured at 0° and 760 mm. Hence the volume of CO present is calculated as

$$\text{Cubic centimeters of CO at } 0°, 760 \text{ mm.} = 0.056 \times \text{N} \times \text{A} \times 1000$$
$$= 56 \text{ N} \times \text{A}$$

where N indicates the concentration of thiosulfate found by standardization, in terms of normality; e.g., if a thiosulfate solution made up as 0.01 N has a factor of 0.965, then N is $0.965 \times 0.01 = 0.00965$. A indicates cubic centimeters of thiosulfate used.

To calculate the volume per cent of CO in the air analyzed the cubic centimeters of CO found in the sample are multiplied by 100 and divided by the volume of the air sample in cc. Therefore,

$$\text{Volume per cent CO in air} = \frac{5600\ \text{N} \times \text{A}}{\text{cubic centimeters of air sample}}$$

The "cubic centimeters of air sample" are calculated by multiplying the volume of air measured in *B* (fig. 14) by the proper factor from table 15 to reduce the volume to 0° .760 mm.

Determination of carbon monoxide in air by the colorimetric method of Sayers and Yant (18)

This method is less exact than the gasometric and titration procedures for CO in air described above, but it is so simple that it can be used for field work, for which it has been employed in the Bureau of Mines. The CO-containing air is equilibrated with blood, in which a proportion of the hemoglobin, dependent on the CO content of the air, is changed to HbCO (see hemoglobin chapter of volume I). The HbCO in the blood is then determined by the colorimetric method of Sayers and Yant.

Reagents

The reagents are those required for determination of CO in blood by the method of Sayers and Yant, as described in the chapter on the determination of hemoglobin and its derivatives.

Procedure

Collecting air sample. A bottle of 250 cc. capacity or larger is filled with water and emptied in the room the air of which is to be analyzed. Or it may be filled with the air by blowing several volumes of the air through the bottle by means of an atomizer bulb. The bottle is closed with a rubber stopper.

Equilibration of air sample with diluted blood. 0.1 cc. of fresh blood is diluted to 2 cc. with water, and is then introduced into the bottle, with the least opening of the latter that is possible. The bottle is then rotated continually for about twenty minutes, without violent shak-

ing or agitation. As much as possible of the inner surface of the bottle should be covered with the blood solution. Every now and then the solution may be thrown centrifugally from the sides to the bottom of the bottle by a quick swinging motion, which allows a new surface to form and aids in reaching equilibrium. When many samples of air are to be analyzed a motor driven equilibrator holding several bottles is a convenience.

Analysis. When the equilibration is finished the blood solution is poured into a test tube, and the percentage of the hemoglobin saturated with CO is determined by the method of Sayers and Yant described in the chapter on determination of hemoglobin and its derivatives.

Calculation. The calculation is carried out according to the equation

$$\frac{\mathrm{HbCO}}{\mathrm{HbO_2}} = a\,\frac{p_{CO}}{p_{O_2}}$$

where p_{CO} and p_{O_2} represent the tensions, or the concentrations, of CO and O_2 respectively in the air, and HbCO and HbO_2 represent the relative concentrations of the indicated hemoglobin compounds in the blood solution. The equation has been fully discussed in the Hemoglobin chapter of Volume I. In place of p_{CO} and p_{O_2} we may write "per cent CO in air" and "per cent O_2 in air," while in place of $\frac{\mathrm{HbCO}}{\mathrm{HbO_2}}$ we may write $\frac{100 - \mathrm{HbCO}}{\mathrm{HbCO}}$, when HbCO is expressed in terms of percentage of total hemoglobin combined with CO. Since the per cent of O_2 in the air is 20.93, the equation may be written

$$\text{Per cent of CO in air} = \frac{\mathrm{HbCO}}{100 - \mathrm{HbCO}} \times \frac{20.93}{a}$$

At the time of Sayers and Yant's work the value of a had not been accurately determined. Sendroy, Liu and Van Slyke (20) have found that for human blood it is 210 and for beef blood 180. Substituting these values, we have

$$\text{Per cent CO in air} = \frac{0.1\ \mathrm{HbCO}}{100 - \mathrm{HbCO}} \text{ when human blood is used or}$$

$$\frac{0.116\ \mathrm{HbCO}}{100 - \mathrm{HbCO}} \text{ when ox blood is used.}$$

Example. HbCO is found to be 30 per cent of the total hemoglobin, human blood being used. Then:

$$\text{Per cent of CO in air} = \frac{0.1 \times 30}{70} = 0.043.$$

BIBLIOGRAPHY

1. Arnold, H. R., Carrier, E. B., Smith, H. P., and Whipple, G. H.: Blood volume studies. V. The carbon monoxide method. Its accuracy and limitations. *Am. J. Physiol.*, 1921, **56**, 313.
2. Bailey, C. V.: Notes on apparatus used in determining the respiratory exchange in man. II. A sampling bottle for gas analysis. *J. Biol. Chem.*, 1921, **47**, 281.
3. Carpenter, T. M.: An apparatus for exact analysis of air in metabolism investigations with respiration exchange chambers. *J. Metab. Res.*, 1923, **4**, 1.
4. Carpenter, T. M., Fox, E. L., and Sereque, A. F.: The Carpenter form of the Haldane gas analysis apparatus. Changes made in the apparatus and details regarding its use. *J. Biol. Chem.*, 1929, **83**, 211.
5. Fieser, L. F.: A new absorbent for oxygen in gas analysis. *J. Am. Chem. Soc.*, 1924, **46**, 2639.
6. Haldane, J. S.: Methods of air analysis. London, 1912, Griffin.
7. Haldane, J. S.: A method of detecting and estimating carbon monoxide in air. *J. Physiol.*, 1895, **18**, 463.
8. Henderson, Y.: Applications of gas analysis. IV. The Haldane gas analyzer. *J. Biol. Chem.*, 1918, **33**, 31.
9. Hill, A. V.: An electrical method (cathetometer) for measuring the CO_2 in respired gases. *J. Physiol.*, 1922, **56**, XX (Proceedings).
10. Higgins, H., and Marriott, W. McK.: A colorimetric method for the determination of the CO_2 percentage in air. *J. Am. Chem. Soc.*, 1917, **39**, 68.
11. Krogh, A.: A gas analysis apparatus accurate to 0.001 per cent, mainly designed for respiratory exchange work. *Biochem. J.*, 1920, **14**, 267.
12. Ledig, P. G., and Lyman, R. S.: An adaptation of the thermal conductivity method to the analysis of respiratory gases. *J. Clin. Invest.*, 1927, **4**, 495.
13. Marriott, W. McK.: The determination of alveolar carbon dioxide tension by a simple method. *J. Am. Med. Ass.*, 1916, **66**, 1594.
14. Newcomer, H. S.: A simple laboratory gas meter and an improved Haldane gas analysis apparatus. *J. Biol. Chem.*, 1921, **47**, 489.
15. Nicloux, M.: Dosage de traces d'oxyde de carbone, dans l'air par la méthode au sang, et considérations générales sur l'absorption de l'oxyde de carbone par l'hémoglobine en l'absence d'oxygène. *Bull. Soc. chim. France,* 1925 (Series IV), **37**, 760.
16. Paal, C., and Hartmann, W.: Die gasvolumetrische Bestimmung des Wasserstoffs durch katalytische Absorption. *Ber. deut. chem. Ges.*, 1910, **43**, 243.
17. Rabinowitch, I., and Bezin, E. V.: A simple and accurate method of determining basal metabolic rates. An electrometric (kathetometer) procedure. *J. Can. Med. Ass.*, 1926, **16**, 638.
18. Sayers, R. R., and Yant, W. P.: The pyrotannic acid method for the quantitative

determination of carbon monoxide in blood and air. Its use in the diagnosis and investigation of cases of carbon monoxide poisoning. Bureau of Mines, Technical paper, 373, 1925, Washington, D. C.

19. SENDROY, J. JR.: The manometric determination of small amounts of carbon monoxide in gas mixtures. *J. Biol. Chem.*, 1932, **95**, in press.
20. SENDROY, J. JR., LIU, S. H., AND VAN SLYKE, D. D.: The gasometric estimation of the relative affinity constant for carbon monoxide and oxygen in whole blood at 38°. *Proc. 13th Internat. Physiol. Congress*, 1929, p. 242.
21. VANDAVEER, F. E., AND GREGG, R. C.: Simplified iodine pentoxide apparatus for determination of carbon monoxide in flue gas. *J. Ind. Eng. Chem.* (Anal. Ed.) 1929, **1**, 129.
22. VAN SLYKE, D. D., AND HANKE, M. E.: Manometric determination of hydrogen and oxygen by combustion, *J. Biol. Chem.*, 1932, **95**, in press.
23. VAN SLYKE, D. D., AND HANKE, M. E.: Manometric determination of hydrogen by absorption with picrate palladium solution. *J. Biol. Chem.*, 1932, **95**, in press.
24. VAN SLYKE, D. D., AND HILLER, A.: Gasometric determination of hemoglobin by the carbon monoxide capacity method. *J. Biol. Chem.*, 1928, **78**, 807.
25. VAN SLYKE, D. D., AND NEILL, J. M.: The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. *J. Biol. Chem.*, 1924, **61**, 523.
26. VAN SLYKE, D. D., AND SENDROY, J. Jr.: Manometric determination of carbon dioxide and oxygen in air. *J. Biol. Chem.*, 1932, **95**, in press.
27. VAN SLYKE, D. D., AND SENDROY, J. Jr.: Carbon dioxide factors for the manometric blood gas apparatus. *J. Biol. Chem.*, 1927, **73**, 127.
28. VAN SLYKE, D. D., SENDROY, J. JR., HASTINGS, A. B., AND NEILL, J. M: Studies of Gas and Electrolyte Equilibria in blood. X. Solubility of carbon dioxide at 38° in water, salt solutions, serum and blood cells. *J. Biol. Chem.*, 1928, **78**, 765.
29. VAN SLYKE, D. D., SENDROY, J. JR., AND LIU, S. H.: Manometric determination of carbon dioxide in air by the isolation method. *J. Biol. Chem.*, 1932, **95**, in press.

CHAPTER IV

Carbon Dioxide and Oxygen Tensions in Alveolar Air

In this chapter the methods will be described which are used to collect the alveolar air for analysis. The methods of analysis have already been outlined in the preceding chapter. Consequently none will be described here, except that of Fridericia, in which the same apparatus is employed both for the collection of the air and for its analysis.

Alveolar air tensions are used chiefly to estimate the tensions of carbon dioxide or oxygen in the arterial or venous blood. The procedures for collecting expired air for measurement of the alveolar tensions of these gases are therefore designed to cause the alveolar air to be in equilibrium, with respect to CO_2, O_2, or both, with either arterial or venous blood at the time the air samples are taken.

Calculation of gas concentrations in terms of tensions

The concentration of a gas, whether pure or in a mixture, is customarily expressed in terms of the *tension or pressure* (the two terms are used synonymously) exerted by the gas at the temperature noted. Thus air under mean atmospheric pressure at sea level exerts a pressure of 760 mm. If, however, the air is saturated with moisture the dry gases are diluted with a certain proportion of water vapor. This proportion depends upon the temperature, which determines the vapor pressure of the water. At 38° the vapor tension of water is 49 mm. of mercury. Hence in air saturated with moisture at 38° under 760 mm. of mercury pressure the *dry gases* will exert only 760 − 49 = 711 mm. pressure. In dry atmospheric air the O_2 tension is 0.2093 × 760 = 159 mm., and is independent of the temperature. If the air is moist, however, and at 38°, the O_2 tension is 0.2093 × 711 = 149 mm. If the barometric pressure were reduced to 380 mm., the O_2 tension in the same moist air at 38° would be 0.2093 × (380 − 49) = 69 mm. And in general if any moist gas mixture is under B mm. of barometric pressure, and the proportion of a given gas in the dry part of the mixture is C volumes per cent (as found by ordinary analysis),[1] the tension, P, of the given constituent gas is calculated as:

$$P = \frac{C}{100}(B - W)$$

[1] See discussion of "Interpretation of gas analyses" in the preceding chapter.

where W represents water vapor tension. For calculating CO_2 and O_2 tensions in alveolar air the value of W is generally assumed to be the vapor tension of water at 38°, or 49 mm. of mercury. For example, if the CO_2 content of a sample of alveolar air is found by analysis to be 5.6 per cent, and the barometric pressure (corrected for temperature) is 756 mm., the alveolar CO_2 tension is calculated as $P_{CO_2} = 0.056\ (756 - 49) = 39.4$ mm.

Gas tension defines concentration only when the temperature is stated, because any gas under atmospheric or other constant tension becomes less concentrated as the temperature rises and the gas expands. "40 mm. of CO_2 tension at 38°" expresses a definite CO_2 concentration, but merely "40 mm. of CO_2 tension" does not.

The concentration of a substance in gas form can be more simply defined in a single figure as moles per liter, in conformity with the usage for solutions. With the gases that are concerned in physiological observations, a mole of dry gas at 760 mm. and 0° occupies 22.4 liters: hence the concentration, of pure O_2, for example, at 0°, 760 mm., is $\frac{1}{22.4} = 0.0446$ molar, or 44.6 millimolar. In general, the concentration of a gas which obeys the gas laws is calculated from the per cent, C, as

$$\text{Millimoles gas per liter} = \frac{C}{100} \times 44.6 \times \frac{B - W}{760\ (1 + 0.00367\ t)}$$

Thus the O_2 concentration in moist air at 38° and 760 mm. is calculated as 7.69 millimolar.

A useful figure to carry in mind, when one is working with gases, is that the concentration of any pure gas at ordinary atmospheric conditions is about M/25.

The employment of molar units for expressing gas concentrations would be a simplication; but convention at present maintains in use pressure at defined temperature.

Barometer correction for temperature

The 760 mm. taken as standard unit of pressure is the pressure which balances a column of mercury 760 mm. high *when the mercury is at 0°*. At warmer temperatures the metal expands so that the same column at 20° would be nearly 763 mm. high. If the brass scale expanded equally with the mercury column the readings would be unaffected by temperature: the barometer would be self correcting. But brass expands only one-tenth as much. One must therefore subtract corrections from all readings made on mercury barometers at temperatures above 0° in order to obtain the height of mercury at 0° which would balance the observed pressure.

The correction is calculated as follows:

The linear coefficient of expansion of mercury is 0.000181 per degree centigrade, that of brass is 0.000019. Therefore, in a mercury barometer with a brass scale the correction for temperature will be $(0.000181 - 0.000019)\ lt = 0.000162\ lt$ where l = the length of the scale involved (in this case the observed barometric reading) and t is the temperature of the barometer in degrees centrigrade.

TABLE 18

EFFECT OF TEMPERATURE ON BAROMETER READINGS AND ON VAPOR TENSION OF WATER

TEMPERATURE	CORRECTIONS IN MILLIMETERS TO BE SUBTRACTED FROM OBSERVED READINGS										VAPOR TENSION OF WATER
	Barometric pressure, mm. Hg, Brass scale					Barometric pressure, mm. Hg, glass scale					
	740	750	760	770	780	740	750	760	770	780	
°C.											*mm.*
15	1.81	1.83	1.86	1.88	1.91	1.92	1.95	1.97	2.00	2.02	12.7
16	1.93	1.96	1.98	2.01	2.03	2.05	2.08	2.10	2.13	2.16	15.5
17	2.05	2.08	2.10	2.13	2.16	2.18	2.21	2.23	2.26	2.29	14.4
18	2.17	2.20	2.23	2.26	2.29	2.30	2.34	2.37	2.40	2.43	15.4
19	2.29	2.32	2.35	2.38	2.41	2.43	2.47	2.50	2.53	2.56	16.3
20	2.41	2.44	2.47	2.51	2.54	2.56	2.60	2.63	2.66	2.70	17.4
21	2.53	2.56	2.60	2.63	2.67	2.69	2.72	2.76	2.80	2.83	18.5
22	2.65	2.69	2.72	2.76	2.79	2.82	2.85	2.89	2.93	2.97	19.7
23	2.77	2.81	2.84	2.88	2.92	2.94	2.98	3.02	3.06	3.10	20.9
24	2.89	2.93	2.97	3.01	3.05	3.07	3.11	3.15	3.20	3.24	22.2
25	3.01	3.05	3.09	3.13	3.17	3.20	3.24	3.29	3.33	3.37	23.6
26	3.13	3.17	3.21	3.26	3.30	3.33	3.37	3.42	3.46	3.51	25.0
27	3.25	3.29	3.34	3.38	3.42	3.46	3.50	3.55	3.60	3.64	26.5
28	3.37	3.41	3.46	3.51	3.55	3.58	3.63	3.68	3.73	3.77	28.1
29	3.49	3.54	3.58	3.63	3.68	3.71	3.76	3.81	3.86	3.91	29.8
30	3.61	3.66	3.71	3.75	3.80	3.84	3.89	3.94	4.00	4.05	31.5

The coefficient of expansion of glass is 0.000008. Therefore, with a glass scale the correction is $(0.000181 - 0.000008)\ lt = 0.000173\ lt$.

Under ordinary barometric conditions the correction for either a brass or glass scale amounts to about $\frac{1}{8}$ mm. for each degree centigrade.

Table 18 gives temperature corrections for mercury expansion in a barometer with either brass or glass scale at temperatures between 15° and 30°C. The vapor tensions of water are also appended to the table.

COLLECTION OF ALVEOLAR FOR DETERMINATION OF ARTERIAL CO_2 TENSION

DISCUSSION

The methods for determining alveolar CO_2 can, in general, be divided into 2 classes: 1, those that aim to determine the CO_2 tension of quickly expelled alveolar air in equilibrium with the arterial blood; 2, those that attempt to determine the CO_2 tension of air retained in the lungs until it is equilibrated with the mixed venous blood from the right ventricle.

Of the methods that aim to determine the tension of gases in the arterial blood, that of Haldane and Priestley (11) has been widely employed. The subject, at rest, breathes normally. When the sample of alveolar air is to be taken, the subject, immediately after completing a normal *inspiration*, makes a quick forced expiration of all the air he can drive out. The last portion expired is taken as "*inspiratory*" alveolar air. "*Expiratory*" alveolar air is obtained by the same technique, except that the forced expiration is begun at the end of a normal *expiration*.

By comparison of CO_2 tension in alveolar air obtained by variations of the Haldane technique with CO_2 tensions calculated from arterial blood analyses, Dill and his associates (8) found, as did Peters, Barr and Rule (20) and Meakins and Davies (18), that in resting subjects the tension of Haldane "expiratory" samples agrees with that of arterial blood better than does the tension of either "inspiratory" samples or the average of "inspiratory" and "expiratory" samples. On the other hand, in exercising subjects "inspiratory" samples proved a more accurate indication of arterial CO_2 tension (9). In rest, apparently, air rapidly expelled after the end of a normal expiration has been in the lungs just long enough to have come into complete carbon dioxide equilibrium with the blood leaving the pulmonary circulation. In exercise the production of carbon dioxide in the tissues is so rapid that CO_2 is able to accumulate in excess in the blood and air during the short time involved in a normal expiration (1).

Experiments by Haldane and Priestley (11), confirmed by later authors (13), have shown that an expiration of 400 cc. will completely wash out the respiratory dead space of a normal resting adult, which is estimated as 125 to 200 cc. Therefore, in such persons all the air expired *after the first 400 cc.* is alveolar air. With an expiration of less than 400 cc., alveolar air can not be secured with certainty.

To circumvent subjective factors which render the original Haldane technique more or less impracticable for clinical use with untrained subjects, various automatic sampling devices have been proposed (8, 12, 14). Some of these appear to yield low values because they introduce an excessive instrumental dead space (8).

Krogh and Lindhard (16) proposed the repeated drawing of small samples of air from the mouth or pharynx at the end of successive normal expirations. Their method has been used to advantage in certain physiological studies, but has found little clinical application for technical reasons. Debenham and Poulton (7) have shown that it yields samples that agree as to CO_2 tension with those collected by the Haldane method.

De Almeida (6) and Pearce (19) calculated the alveolar CO_2 tension from the content of CO_2 in the total expired air and an assumed dead space value. The uncertain size of the dead space appears to make this calculation subject to greater errors than the usual calculation based on the CO_2 content of the last portion of air expired.

Of the methods of collecting air for determining arterial CO_2 tension, we describe below that of Haldane as applied to normal resting subjects, its modification by Henderson and Morris, and that of Fridericia, which has over others the advantage that the sample of alveolar air is both collected and analyzed in a single simple piece of apparatus.

HALDANE AND PRIESTLEY'S METHOD OF COLLECTING AIR FOR ARTERIAL CO_2 TENSION (11)

The *apparatus* (fig. 15, *1*) required is a straight tube of 2 cm. bore, and about 1 meter in length, with a capillary side tube near one end for withdrawal of air samples. Haldane and Priestley used a smooth hose about 4 feet long, without valve or mouth-piece. At the end of the forced expiration the end of the tube was closed by the tongue of the subject until an air sample had been withdrawn for analysis through a side arm in the tube. This method is impracticable for untrained subjects. For this reason Boothby and Peabody (3) interposed a three-way valve (fig. 15, *B*) between the tube and the subject. The valve is so constructed that the subject, with the end of the tube in his mouth, may breathe outside air unrestrictedly until the moment when it is desired to secure a sample; then it must be possible to connect him almost instantaneously with the tube. The connection with the tube must be straight and smooth to avoid the production of eddies and currents. The dead space formed by the valve and its connections with the patient must be as small as possible. The collecting tube, as near the valve as possible, is pierced by a small aperture communicating with a fine tube by means of which samples of air may be withdrawn for analysis.

Procedure

The apparatus is assembled as shown in figure 15, 1. The gas sampler, *F*, is connected to the side tube, which is completely filled with

mercury up to the breathing tube. The stop-cock of the sampler is closed and slight negative pressure is produced in the sampler by lowering the leveling bulb. The breathing tube, with a mouth-piece if necessary, is inserted in the subject's mouth with the three-way valve,

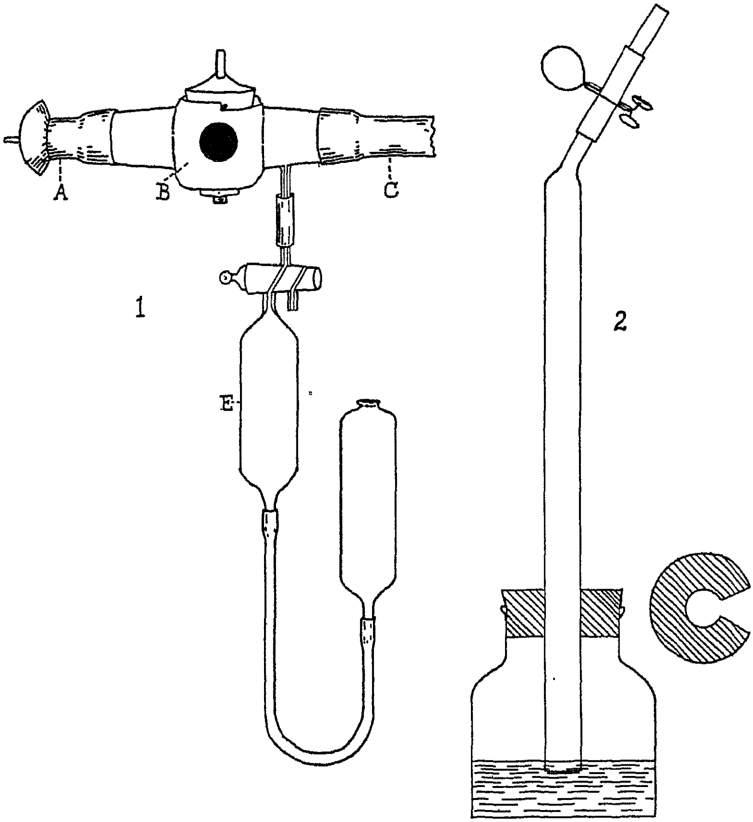


FIG. 15. Apparatus for the collection of Haldane alveolar air samples. 1. Usual apparatus. *A*, mouth-piece; *B*, three-way valve; *C*, collecting tube, about 1 meter in length; *D*, capillary sampling tap with sampling tube *E* attached. 2. Apparatus of Henderson and Morriss (14).

B, turned to connect him with the outside air. A nose-clip may be used, but it is likely to disturb the respirations and should therefore be avoided if possible. When the subject is breathing normally and regularly, just at or after the beginning of expiration the valve is turned so that he is connected with the tube ***C***. He is then directed to blow out hard. At

the very end of expiration the valve is returned to its original position and a small sample of air is immediately withdrawn by opening the stop-cock of the sampler for a moment. The sample is then analyzed for carbon dioxide (and oxygen, if desired).

Precautions. The chief sources of error in the method are subjective. The operator must be so highly trained that his motions can be made with rapidity, and yet so quietly and skilfully that they do not disturb the subject. Each motion must be made at exactly the proper stage of the respiratory cycle. The respirations of the subject must remain natural throughout the procedure. The tendency to catch a short breath just before the forcible expiration must be prevented. The expiration must be made entirely through the mouth, which entails voluntary occlusion of the nasal passages (manual occlusion of the nostrils or the use of a nose-clip, though undesirable, is sometimes necessary). Finally, the valve must be closed sharply at the end of the expiration to prevent even the smallest inspiratory motion from contaminating the sample with room air. A certain amount of practice is required before even the most intelligent subject can deliver good alveolar samples. Duplicate samples from separate expirations should differ by no more than 0.2 per cent of CO_2, which is equivalent to a little over 1 mm. of CO_2 tension.

HENDERSON AND MORRISS' (14) METHOD OF COLLECTING AIR FOR ARTERIAL CO_2 TENSION

To obviate some of the subjective sources of error in the Haldane method of collecting samples Henderson and Morriss proposed the use of the apparatus illustrated in figure 15, *2*. This consists of a vertical tube of 50 to 100 cc. capacity held by a cork, from which a segment has been removed, in a small wide-mouthed bottle containing acidulated water. The distal end of the tube projects just beneath the water, and thus forms a Mueller valve. The other end of the tube is contracted to 7 or 8 mm. inner diameter and connects, by means of a piece of rubber tubing, with a short piece of glass tubing that serves as a mouth piece. The rubber connecting tube is closed by means of a pinch-cock.

During a normal expiration the pinch-cock is opened and the mouth-piece is inserted between the lips of the subject who is then directed to prolong the expiration forcibly. Just at the end of the expiration the pinch-cock is closed. The glass mouth-piece is replaced with a sampler, the lower end of the tube is immersed beneath the water, and a sample

of air is withdrawn from the upper part of the tube. One should draw the sample at once, before CO_2 is lost by diffusion into the water. The same precautions cited above for the Haldane-Priestley method are, of course, necessary.

The Mueller valve serves a double purpose. It prevents entrance of room air into the tube, and any inspiration at the beginning or end of the expiration can be detected by observation of the water column in the tube.

It is quite possible to utilize the Mueller water valve also in the Haldane technique. In this case the tube can be made much shorter (to contain not more than 100 cc.) and of rigid material, metal or glass. It need not be absolutely vertical if the lower end is cut off at an angle. It should not project more than a few millimeters below the surface of the water, to avoid any serious obstruction to the flow of air. For the same reason the segment removed from the stopper of the bottle must be large enough to afford for the escape of air an opening equal to the cross section of the collecting tube.

FRIDERICIA'S APPARATUS FOR BOTH COLLECTION AND ANALYSIS OF AIR FOR ARTERIAL CO_2 TENSION (10)

Fridericia has devised a simple apparatus that serves for both collection and analysis of alveolar air samples. It has received wide clinical application, for which it is probably the method of choice when the subject can cooperate to the extent of blowing into the apparatus as directed. Theoretically it suffers from disadvantages in comparison with the Haldane method in that the 4-mm. cocks of the Fridericia apparatus slightly impede the expiration, and the accuracy of the CO_2 determination yielded by the apparatus (0.1 per cent) is not equal to that of the Haldane and manometric methods that can be used when air is collected by the apparatus shown in figure 15, *E*. However, these differences combined hardly affect results by 1 mm. of CO_2 pressure, which is less than the variation that may be encountered in successive determinations on a normal subject by the more precise methods.

The *apparatus* is shown in figure 16. The 120-degree three-way stop-cock *A* can either connect the mouth-piece tube with the gas-burette *C*, or can connect either arm with the bottom outlet *D*. The burette from (and exclusive of the bore of) stop-cock *E* to (and inclusive of the bore of) stop-cock *A* has a capacity of 100 cc. About 90 cc. of this is in the bulb; the remaining 10 cc. is in the tube, which is calibrated in divisions each of which represents 0.1 per cent of the total capacity of the burette.

A glass jar is required, large enough to hold the apparatus in the vertical position with the bulb of the burette submerged in water.

Reagents

Sodium hydroxide, 10 per cent.
Acetic acid, 5 per cent, *or boric acid,* saturated solution.

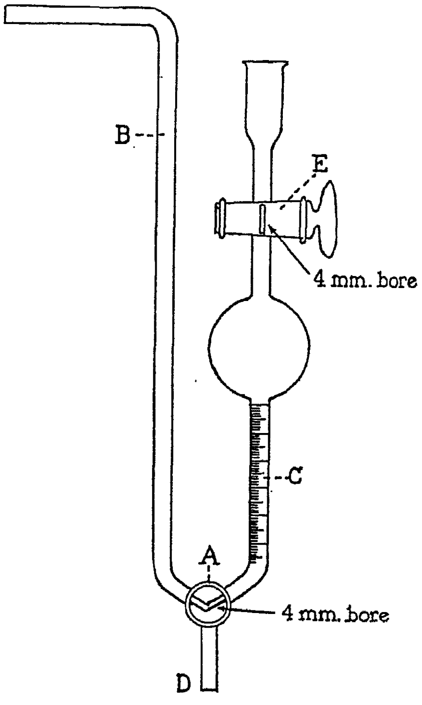


FIG. 16. Fridericia (10) tube for the determination of alveolar CO_2

Procedure

The apparatus is rinsed with 5 per cent acetic acid before it is used. With stop-cock *E* open and cock *A* connecting *B* with *C*, the apparatus is held with the mouth-piece *B* near the lips of the subject. At or just after the beginning of a normal expiration the mouth-piece is placed between the lips of the subject, who is then directed to blow out hard. At the very end of the expiration stop-cock *E* is closed and the tube re-

moved from the subject's mouth. The apparatus is then placed in the glass jar, with the bulb entirely submerged in water at room temperature. When the gas has cooled to the temperature of the water, which requires three to five minutes, stop-cock *A* is turned to a neutral (closed) position. The apparatus is then taken out of the bath and 10 per cent sodium hydroxide is pipetted into *B* until tube *B* is filled almost to the elbow.[2] Cock *A* is again opened to connect *B* and *C*, whereupon the pressure of the fluid column in *B* causes a small quantity of the alkali to flow into the burette. With the cock in the closed (neutral) position again, the apparatus is turned to a horizontal position. The alkali in *C* runs into the bulb of the burette and is gently shaken around to promote absorption of the CO_2. The apparatus is then turned upright again and more alkali admitted to replace the volume of CO_2 that has been absorbed. The apparatus is again shaken as before. The procedure is repeated if necessary until there is no further visible absorption of gas. Then cock *A* is turned to connect *B* and *C* and the apparatus is immersed in the bath. The column of fluid in *C* moves either up or down until the temperature of the gas reaches that of the bath.

Now one must lower the level of the column of fluid in *B* to the level of that in *A* in order to read the latter under atmospheric pressure. Cock *A* is closed, the apparatus is lifted from the bath and held upright, and alkali solution from *B* is run out through *D* until the meniscus in *B* has fallen to the level of that in *C*. Cock *A* is then turned to reconnect *B* and *C*, and the apparatus is again immersed in the bath. If the column of alkali remains stationary, with the menisci in *B* and *C* at the same levels, the per cent CO_2 is read on the scale of *C*, and the analysis is finished. Frequently, however, a slight shift of the alkali column occurs after immersion in the bath, and a little alkali must be removed from or added to *B* to equalize the final levels in *B* and *C*.

The final level adjustment described in the above paragraph can be replaced by the following simpler technique. Without removing the apparatus from the bath a narrow rubber tube, attached to a pipette, is slipped into *B*, and alkali solution is sucked out of *B* until the liquid levels in *B* and *C* are equal.

[2] The alkali should be introduced so that it flows down the sides of the tube, displacing the gas cleanly from below upward. Fridericia recommended that the alkali be admitted through *D*. This necessitates sucking on the mouth-piece or immersing the apparatus in alkali. If this procedure is followed the bore of the stop-cock must not be included in the capacity of the burette.

After the analysis is finished the apparatus is washed out with water and 5 per cent acetic acid.

The CO_2 tension is calculated from the per cent and the barometric pressure by the equation on page 161.

Collection of Alveolar Air for Determination of Venous CO_2 Tension

DISCUSSION

In all methods of collecting alveolar air for determination of venous CO_2 tension the subject rebreathes a limited volume of air until its CO_2 tension rises to that of the venous blood coming to the lungs. The requirements (discussed in the acid-base balance chapter of volume I) of an accurate procedure for the determination of the CO_2 tension of mixed venous blood are the following:

1. There shall be no abnormal hindrance to diffusion of CO_2 from the blood to alveolar spaces.
2. No blood which has been aerated in the lungs shall again return to the pulmonary circulation after traversing the systemic circulation during the time consumed by rebreathing. The rebreathing can not, for precise results, be continued beyond 15 or 20 seconds (2, 15).
3. A complete and uniform mixture of gases must be attained throughout the lungs and rebreathing apparatus. The volume of air rebreathed must be sufficiently small in comparison with the rate of carbon dioxide production to permit the attainment of carbon dioxide equilibrium between air and pulmonary blood in the period permitted for rebreathing.
4. Because of the effects of oxygen upon the carbon dioxide carrying power of the blood, the oxygen tension in the lungs and rebreathing system must, for the most precise results, approximate that of the mixed venous blood when the sample of alveolar air is collected.

The original method of Plesch (21) is the simplest of the rebreathing procedures. It has been extensively used for clinical purposes because, of all available methods, it alone requires no cooperation on the part of the subject and can therefore be employed even with comatose patients or infants. It consists essentially of rebreathing air from a bag for fifteen to thirty seconds. The CO_2 tension of the rebreathed air may fail in this time, especially when respirations are shallow, to rise quite up to venous tension. On the other hand the O_2 tension in the lungs remains higher than venous, and must tend to raise a little the CO_2 tension, because of the mutual tendency of O_2 and

CO_2 to push each other out of the blood (see hemoglobin chapter, volume I). The two effects tend to balance each other. The Plesch method, as an approximate measure of venous CO_2 tension, has proven satisfactory in wide clinical application, especially in studies of acidosis, where significant changes are large ones.

Christiansen, Douglas and Haldane (5) used for inhalation, from the start of the rebreathing, mixtures of CO_2 and O_2 of about the tensions expected. After one rebreathing the gas mixture was again analyzed. If its composition had changed, a new mixture was prepared. This method of trial and error was continued until a mixture was attained which could be rebreathed for a period less than the estimated duration of one circuit of the circulation without appreciable change of its composition. The CO_2 and O_2 tensions in this mixture were considered to equal those in the venous blood.

The procedure was laborious and time consuming. Henderson and Prince (15) modified it by introducing repeated, intermittent rebreathing of the same portion of air. They found that the CO_2 tension of a gas mixture, if rebreathed at intervals for periods less than the duration of a circuit of the circulation, approached a constant level, regardless of whether the CO_2 tension of the initial mixture was higher or lower than that of the blood. This level they considered to be at least approximately that of the venous blood. They thus used a series of gas mixtures which progressively approximated in CO_2 content the composition of "venous alveolar air." The O_2 tension, as shown by Burwell and Robinson (4), remains above that of venous blood, but the CO_2 tension is not significantly different from that obtained when the O_2 is regulated, as described below, in Burwell and Robinson's method for venous O_2 tension.

In order to obtain venous oxygen tensions by either continuous or intermittent rebreathing it is necessary to start with air of very low oxygen content. Barcroft, Roughton, and Shoji (2) in fact obtained good results (30 to 40 mm. O_2 tension) by rebreathing pure nitrogen gas by the Plesch technique. Four or five normal breaths were taken to and from the bag of nitrogen during a period not exceeding twenty seconds. The third, fourth, and fifth expirations showed constant O_2 content. After twenty seconds the O_2 content began to fall rapidly, indicating that a second circulatory cycle had started,

Burwell and Robinson (4) used intermittent rebreathing. They washed out the lungs by preliminary inhalation of a mixture of N_2 with about 1 per cent of O_2 and 6 per cent of CO_2. Thereby they were able to obtain alveolar air which approximated the mixed venous blood in the tension of O_2 as well as CO_2. The method requires intelligent cooperation on the part of the sub-

ject under examination. The accuracy of the results for venous CO_2 tension obtainable from patients with cardiac and respiratory disorders is uncertain. Burwell and Robinson review previous methods for obtaining alveolar air of venous CO_2 and O_2 tensions.

CONTINUOUS REBREATHING METHOD OF PLESCH FOR VENOUS CO_2 TENSION (21)

Apparatus. A bag of about 1500-cc. capacity is the only apparatus required. It is convenient to have it fitted with a valve and mouth-piece as illustrated in figure 15, *1*. The rubber bag replaces the collecting tube shown in figure 15, *1*, c. If no valve is at hand, one can use a bag provided merely with a wide rubber tube inlet, which serves as mouth piece and is opened and closed by an ordinary pinch clamp.

Procedure

The bag is filled with about 1000 cc. of air for an adult, or 600 cc. for an infant (17). The subject, with his nose clamped and the mouth-piece (fig. 15, 1) inserted, breathes outside air for a short period. At the end of an expiration he is connected with the bag. He then re-breathes the mixture in the bag for twenty-five seconds. If the subject can cooperate, he is told to breathe somewhat more deeply than usual, and about five respirations suffice. At the end of the period the valve is closed and a sample of gas is withdrawn from the bag and analyzed for CO_2. The sample should be taken from the bag as soon as the period is over, because CO_2 can escape rather rapidly by diffusion through the rubber walls of the bag.

If one uses the Higgins-Marriott method, described in the preceding chapter, for analyzing the air, a glass tube drawn out to a capillary is attached to the outlet of the bag. The capillary is dipped into the test tube of 0.01 N NaOH used in the method, and the bag is pressed so that the gas bubbles through the solution.

If the subject is unconscious or entirely unable to cooperate, a tight-fitting mask must be substituted for the mouth-piece and nose-clip. In this case it is impossible to influence the type of breathing. If the respirations are shallow, the CO_2 tension found may be too low.

INTERMITTENT REBREATHING METHOD FOR VENOUS CO_2 TENSION. HENDERSON AND PRINCE (15); BURWELL AND ROBINSON (4)

Apparatus. This consists, as in the Plesch method, of a rubber rebreathing bag with a capacity of about 1500 cc. attached to a three-way valve (see figure 15, *1*) with a mouth-piece.

Procedure

The mouth-piece is placed in the subject's mouth with the valve turned so that he is connected with the outside air. A nose-clip is applied. At a given signal he draws in a full breath. The valve is immediately turned to connect him with the empty bag, into which he exhales as far as possible. Communication is then shifted by means of the valve back to outside air. After an interval of about three minutes to allow respirations to become normal again, and at the end of a complete forced expiration, he is again connected with the bag. He breathes in and out forcefully from the bag three times, ending as before with a complete expiration, after which he is again connected with the outside air. This procedure is repeated at three-minute intervals from four to six times, if the subject is capable of fairly deep respiration. Otherwise repetition is continued until samples from the bag after two successive rebreathing periods check within 0.2 per cent in CO_2 content. It is advisable for the subject to release himself from the mouth-piece during the intervals between rebreathing periods.

COLLECTION OF ALVEOLAR AIR FOR DETERMINATION OF VENOUS TENSIONS OF BOTH CO_2 AND O_2. METHOD OF BURWELL AND ROBINSON (4)

The *apparatus* is arranged is shown in figure 17 and its legend.

Procedure

The nose of the subject is closed with a nose-clip and he applies his mouth to the breathing tube and expires fully, the cock being turned so that the expiration escapes through F to the outside air. He then takes five full breaths. During the first two inspirations the cock remains in position so that air is inspired from the spirometer at G and expired through F. At the end of the expiration following the second inspiration from the spirometer the cock is turned, so that the third, fourth and fifth breaths are in and out of the bag at C. At the end of the last expiration into the bag the cock is again turned, shutting off the bag, and the subject is directed to remove his mouth from the breathing tube and to breathe outside air. The entire procedure requires from eighteen to twenty seconds, divided between the inspirations from the spirometer and the rebreathing to and from the bag. The procedure can be carried out correctly by ordinary hospital patients with little or no practice. The subject is fairly dyspneic for five or six breaths after the rebreathing and may show moderate cyanosis, but there is no change in heart rate. This procedure is repeated seven to eleven times, with an interval of

three minutes between each, until the O_2 tensions cease to show significant change. The gas samples for analysis are taken from the bag immediately after each rebreathing so that diffusion of gases through the rubber bag does not affect the results.

The total duration of each rebreathing period must not exceed the duration of a complete circulatory cycle. This time, though usually about fifteen or twenty seconds (2, 15) doubtless varies in different people and under different circumstances. The technique prescribed is

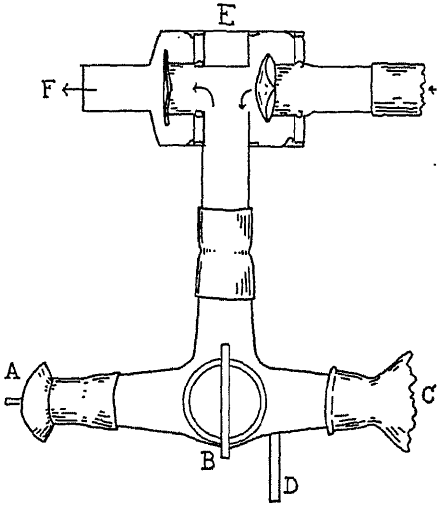


FIG. 17. Apparatus of Burwell and Robinson (4) for obtaining alveolar air with venous CO_2 and O_2 tensions. *A*, mouth-piece; *B*, three-way cock; *C*, rebreathing bag; *D*, sampling tap; *E*, two-way Rosling valves in series; *F*, expiratory outlet to room; *G*, inspiratory tube connecting with a 100-liter spirometer containing nitrogen gas plus 1 per cent of O_2 and 5 per cent of CO_2. Bag *C* is empty at the start of an experiment.

suitable to normal, resting adults. In exercise, presumably, the duration of rebreathing should be curtailed. The influences pathological conditions may have on the permissible length of the periods have not yet been ascertained.

BIBLIOGRAPHY

1. AITKEN, R. S., AND CLARK-KENNEDY, A. E.: On the fluctuation in the composition of the alveolar air during the respiratory cycle in muscular exercise. *J. Physiol.*, 1928, 65, 389.
2. BARCROFT, J., ROUGHTON, F. J. W., AND SHOJI, R.: The measurement of the oxygen

content of the mixed venous blood, and of the volume of blood circulating per minute. *J. Physiol.*, 1921, **55**, 371.

3. Boothby, W. M., and Peabody, F. W.: A comparison of methods of obtaining alveolar air. *Arch. Int. Med.*, 1914, **13**, 497.
4. Burwell, C. S., and Robinson, G. C.: A method for the determination of the amount of oxygen and carbon dioxide in the mixed venous blood of man. *J. Clin. Inv.*, 1924, **1**, 47.
5. Christiansen, J., Douglas, C. G., and Haldane, J. S.: The absorption and dissociation of CO_2 by human blood. *J. Physiol.*, 1914, **48**, 244.
6. de Almeida, M. O.: Sobre los principios para un novo methodo de determinaco da tensao de CO_2 no ar alveolar. *Brazil Medico.*, 1916, **30**, 303.
7. Debenham, L. S., and Poulton, E. P.: The respiration and oxyhaemoglobin dissociation curves and buffer value of the blood in normal men. *Quart. J. Med.*, 1918–19, **12**, 38.
8. Dill, D. B., Hurxthal, L. M., van Caulaert, C., Fölling, A., and Bock, A. V.: The carbon dioxide equilibrium in alveolar air and arterial blood. II. Resting. *J. Biol. Chem.*, 1927, **74**, 303.
9. Dill, D. B., Lawrence, J. S., Hurxthal, L. M., and Bock, A. V.: The carbon dioxide equilibrium in alveolar air and arterial blood. III. Exercising subjects. *J. Biol. Chem.*, 1927, **74**, 313.
10. Fridericia, L. S.: Eine klinische Methode zur Bestimmung der Kohlensäurespannung in der Lungenluft. *Berl. klin. Woch.*, 1914, **27**, 1268.
11. Haldane, J. S., and Priestley, J. G.: The regulation of the lung-ventilation. *J. Physiol.*, 1904–05, **32**, 225.
12. Henderson, Y.: Automatic sampling of alveolar air, particularly for measurement of the circulation with ethyl iodide vapour. *J. Physiol.*, 1926–27, **62**, 262.
13. Henderson, Y., Chillingworth, F. P., and Whitney, J. L.: The respiratory dead space. *Am. J. Physiol.*, 1915, **38**, 1.
14. Henderson, Y., and Morriss, W. H.: Applications of gas analysis. I. The determination of CO_2 in alveolar air and blood, and the CO_2 combining power of plasma, and of whole blood. *J. Biol. Chem.*, 1917, **31**, 217.
15. Henderson, Y., and Prince, A. L.: Applications of gas analysis. II. The CO_2 tension of the venous blood and the circulation rate. *J. Biol. Chem.*, 1917, **32**, 325.
16. Krogh, A., and Lindhard, J.: The volume of the "dead space" in breathing. *J. Physiol.*, 1913–14, **47**, 30.
    On the average composition of the alveolar air and its variations during the respiratory cycle. *J. Physiol.*, 1913–14, **47**, 431.
    The volume of the dead space in breathing and the mixing of gases in the lungs of man. *J. Physiol.*, 1917, **51**, 59.
17. Marriott, W. McK.: The determination of alveolar carbon dioxide tension by a simple method. *J. Am. Med. Ass.*, 1916, **66**, 1594.
18. Meakins, J., and Davies, H. W.: Observations on the gases in human arterial and venous blood. *J. Path. Bact.*, 1920, **23**, 451.
19. Pearce, R. G.: Studies in the physiology of the respiration. I. The capacity of the air passages and the percentage of carbon dioxide in the alveolar air during rest and exercise. *Am. J. Physiol.*, 1917, **43**, 73.
20. Peters, J. P., Barr, D. P., and Rule, F. D.: I. The carbon dioxide absorption curve and carbon dioxide tension of the blood of normal resting individuals. *J. Biol. Chem.*, 1921, **45**, 489.
21. Plesch, J.: Hämodynamische Studien. *Z. exp. Path. Therap.*, 1909, **6**, 380.

CHAPTER V

Respiratory Metabolism

GENERAL CONSIDERATIONS CONCERNING RESPIRATORY APPARATUS[1]

For the determination of the exchange of oxygen and carbon dioxide two general types of apparatus have been employed, the so-called "open-circuit" and "closed-circuit" types.

Open-circuit apparatus

In the open-circuit types of apparatus, atmospheric air is inspired by the subject, and the expired air is analyzed for CO_2 or O_2, or both. The total volume of air breathed is also measured. From the data thus secured the amount of oxygen absorbed and the amount of carbon dioxide given off in a given period can be calculated.

In earlier apparatus ingoing and outgoing air were measured by some device, usually accurately calibrated gas meters, and samples of the outgoing air were analyzed at intervals. In the Tissot (40) apparatus, described later in detail, which has now largely displaced other open-circuit machines for experiments on resting subjects, the total volume of air expired in the experimental period is collected in an accurately balanced and calibrated spirometer. Samples of the air are then analyzed for carbon dioxide and oxygen.

For experiments on exercising men Douglas (25) substituted for the Tissot spirometer a large gas bag which can be carried by the experimental subject. At the end of the period of investigation the expired air in the bag is analyzed for carbon dioxide and oxygen, and is measured by passage either through a gas meter or into a spirometer. The Douglas bag has attained nearly universal use for observations on working subjects.

Closed-circuit apparatus

In closed circuit types of apparatus the subject inspires from and expires into an enclosed body of air or oxygen. The CO_2 is removed by alkali absorbent and the O_2 consumption is measured by various means.

[1] For general discussions of total metabolism, the technique for its determination, and the effect upon it of physiological and pathological factors, the reader is referred to works cited in references 8, 12, 13, 16, 17, 18, 27 and 36 in the bibliography, and to Chapter 1 in Volume I.

McClendon, Hilding, Steggerda, Conklin and Whitaker (37) have described a closed circuit apparatus consisting of two spirometers of the Tissot type immersed in a large common water bath. The subject inhales from one of these and exhales into the other. The volumes of air in both are determined before and after the respiratory period. The expired air is then returned to the first spirometer through a by-pass in which it is free from carbon dioxide by exposure to soda-lime. Both O_2 consumed and CO_2 produced can be calculated from the successive changes in the volumes of air in the two spirometers. The apparatus is large and expensive, but the procedure is simple. The same is true of a somewhat similar apparatus of Hagedorn (28) which will be described later.

The closed-circuit apparatus which has been employed most in this country for clinical purposes is that of Benedict (7, 22). In this the subject rebreathes air constantly from a system of tubes and bottles in which a circulation is maintained by means of a pump. The oxygen in the system is constantly renewed from an oxygen tank, while the carbon dioxide is removed by soda-lime. The oxygen consumed and the carbon dioxide produced are estimated from the changes in weight of the oxygen tank and the soda-lime containers and the change in volume of air in the system. The last is measured by means of a recording spirometer. The Benedict apparatus requires less time for both operation and calculation than the open-circuit machines like the Tissot, which require gas analyses. With the aid of one technician Carpenter (22) was able "to make a series of three 15-minute periods and to calculate the results in the minimum time of an hour and a half. . . . . In a long series of experiments it was possible in every case to complete the calculations of the first two 15-minute periods by the end of the third period." This is an important consideration, especially in experiments in which the immediate results of some treatment are to be investigated and one wishes to be certain that the metabolism in advance of the experiment is at a constant level. Nevertheless, for combined observations of CO_2 output and O_2 intake, the original Benedict apparatus has largely given way to the Tissot open-circuit type of apparatus for clinical work, chiefly because the smaller number of connections in the Tissot makes it relatively immune to leaks.

Tubing, valves, masks, and leaks

Tubing of respiratory apparatus, and likewise valves, intended for the use of adults should have a lumen with a cross area of at least 6 cm². Round tubing of this size has a bore of 2.5 cm. (1 inch). Where flexibility is required non-collapsible tubing of the "gas-mask" type is to be preferred be-

cause it does not become obstructed when it is bent. Such tubing can be purchased from most dealers in respiratory apparatus or physiological equipment. Hose reinforced inside by coiled metal is equally good for most purposes, but is heavier and less flexible. Brass connecting tubes are superior to glass for rigid parts because brass is less fragile. For small children and infants, tubing of 2 cm. ($\frac{3}{4}$ inch) diameter is satisfactory.

Two and three-way valves of aluminum or brass must turn easily and still be so firmly ground that they will not leak, and must be of smooth bore throughout. Aluminum cocks of this type are illustrated in figure 15, 1, *B*, and figure 17, *B*, (pp. 166 and 175).

Innumerable *valves*, with individual advantages, have been devised *to permit the passage of air in only one direction.* Desirable features in such valves are: low resistance, quick action, absence of "back-lash," small dead space, and ability to work in any position. From the standpoint of resistance, quick action and absence of "back-lash," the Müller water valve, illustrated in figure 15, *2*, (p. 166) is almost ideal. However, such valves have limited value because of their large dead space and the fact that they must be kept always in a vertical position. The latter objection applies to all valves which depend upon the force of gravity for closure. Since the experience with gas-masks during the war, extremely efficient rubber valves of various kinds have become available. Of these the most useful are the Rosling (fig. 18, *1*) and the "flutter" (fig. 18, *2*) types. The latter is, perhaps, superior from the point of view of speed of action and absence of "back-lash," but requires a larger dead space for casing. The Rosling valve consists of two squares of rubber, attached to one another only at the corners. One of the rubber squares is perforated by a 1-inch hole to which is sealed a short piece of 1-inch rubber hose. This valve is almost, if not quite, as efficient as the flutter valve and has less dead space. It has, therefore, become extremely popular in respiratory apparatus. A pair of these valves in metal casing is illustrated in figure 17, *E* (p. 175).

For most purposes, especially for respiratory studies of short duration with cooperative subjects, a *mouth-piece* is preferable to a mask. The necessity of breathing through the mouth, however, causes most subjects at first to overventilate. Some individuals do not overcome this tendency, or they salivate excessively, compelling the use of a mask. Numerous *spring nose clips* are available on the market, of which the most generally useful are those equipped with a ratchet or other device by which they can be locked in place. Mouth-pieces of the type illustrated in figure 18, *3*, can be obtained in different size and are adaptable to almost any subject who is

not entirely toothless. Occasionally it is necessary, especially in toothless subjects, to hold the corners of the lips in place over the mouth-piece with strips of adhesive plaster.

Face masks belong to two general types: 1, similar to gas masks and enclosing the whole face or even the head; 2, half-masks, enclosing only the nose and mouth, and made adaptable by inflated rubber edges. These are fastened to the face with two or more straps. Both types of mask, but especially the gas mask, furnish a larger dead space than does the mouth-piece. Besides this they are more prone to leak.

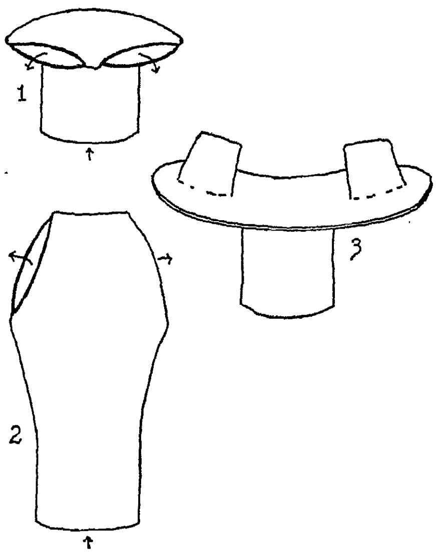


FIG. 18. *1*, Rosling rubber valve; *2*, rubber flutter valve; *3*, rubber mouth-piece

In all masks and mouth pieces the *dead space* into which the subject rebreathes (the volume of the apparatus between the mouth or nose and the intake valve) must be as small as possible. In this space carbon dioxide is left after each expiration to be inhaled with the following inspiration. If the dead space is large the amount of carbon dioxide in the inspired air may become great enough to stimulate the respiration.

Testing respiratory apparatus for leaks. The elimination of leaks about a mask or mouth-piece or from the clamped nose is sometimes a matter of difficulty. If the leak is large enough it may be detected by means of a cold mirror or polished steel sheet, which becomes fogged by the hot moist air escaping from the leak during expiration. In case of uncertainty applica-

tion of shaving lather to the doubtful points is a useful expedient. If there is a leak bubbles are seen coming through the lather.

The general principles involved in the detection of leaks are always the same. With the apparatus as a whole closed, the gas within it is subjected to pressure and any change of volume noted. Alterations of temperature must always be taken into account. If it is certain that the apparatus as a whole leaks, individual portions of the apparatus are subjected to similar tests. If this is impossible various parts of the apparatus which are under suspicion may be immersed in water or lathered with shaving soap to detect bubbling. Often enough, ingenuity fails, and it becomes necessary to dismantle an apparatus completely, and test and reassemble it piece by piece.

CONSTRUCTION OF SPIROMETERS

Spirometers which are employed for the collection of air in respiratory studies usually belong to one of two types: the bell type (figs. 19 and 21) or the bellows type (fig. 22). The movable chambers of both are commonly suspended in water, which acts as a seal against escape of gas. Occasionally other fluids in which air and its component gases are less soluble are used. It is usually necessary to know the temperature of the gas in the spirometer. For this purpose a thermometer is inserted in the dome of the bell.

The chief requisites of a spirometer for most purposes are: 1. It shall move with the least possible resistance. 2. Changes in volume of the gas in the spirometer shall bear a linear relation to the motions of the spirometer, so that air intake is directly proportional to rise of spirometer bell.

If the expired air collected in the spirometer is to be analyzed the following additional requirements must be met. 3. Change of gas composition by diffusion of CO_2 into the water use as sealing fluid must not be appreciable. 4. Gases entering the spirometer must be able to mix so readily that gas layers of different composition do not form. 5. The spirometer when empty must have a dead space so small that the gas trapped in it can be readily washed out.

1. *Absence of resistance.* The entrance of expired air must cause the bell to rise without more pressure difference between the air inside and outside the bell than is indicated by a water column a few millimeters high. A water manometer may be attached to the intake tube of the spirometer.

A *counterpoise* is used to balance exactly the weight of the bell at all positions. The adjustment of the counterpoise is an important point which is discussed in a separate section below.

To minimize resistance from *inertia due to weight* the spirometer bell is made as light as is compatible with rigidity.

Friction is reduced to a minimum by careful alignment of movable parts and the use of high class bearings, well lubricated. The water seal is made wide enough to permit the bell to rise and fall in it freely, and the bell is carefully aligned to prevent it from coming into contact with the metal on either side of the water seal.

Even in a spirometer perfectly made and set up, resistance to respiration may at times occur from adhesions of valve leaflets, caking of soda lime, kinking of tubes, or collection in the tubing of water from condensation of vapor. Such obstructions may become manifest by either a gradual or a sudden increase in resistance. The location of the obstruction depends on the ingenuity of the operator.

2. *Constant ratio of bell rise to volume increase.* In order that changes in volume of the gas in the spirometer shall bear a linear relation to the motions of the spirometer bell, the latter must be of uniform internal cross section area throughout its length. This is a matter which rests entirely with the manufacturer. If, when the bell is calibrated as described later, significant variation in the size of the bell at different levels is found, a correction curve must be prepared.

3. *Prevention of CO_2 diffusion into water.* To prevent measurable absorption of CO_2 by the ring of water seal to which the gas in the spriometer is exposed, the ring should be as narrow as it can be, and still permit perfectly free movement of the bell as it rises and falls. The water level should never rise into the conical dome of the inside shell. As a further provision against CO_2 absorption it is well to keep the spirometer, when not in use, filled with gas approximating in CO_2 content the mixture which one intends to measure or store in the instrument. In the Tissot apparatus for the determination of respiratory metabolism, some of the expired air is allowed to remain in the spirometer after each determination. This keeps the water in the seal partially saturated with gas of the proper composition. As an additional precaution under unusual circumstances some fluid, such as glycerol or nearly saturated calcium chloride solution, in which CO_2 is much less soluble than in water (see footnote, page 86, chapter III), could be substituted for the latter in the seal, but in practice this precaution has not been found necessary. Salts tend to cause corrosion of the metal.

4. *Avoidance of layering.* If the gas entering a spirometer formed layers of varying composition it would have to be mixed before a representative sample could be drawn. However, Carpenter (24) has found by tests that, with the Tissot spirometer, the motion with which expired air enters the bell is sufficient to assure mixing. When artificial gas mixtures are mixed in a spirometer a fan may be introduced into the apparatus.

5. *Minimizing dead space.* To minimize dead space in the Tissot type of spirometer the water seal and the water level are so adjusted that when the spirometer is empty the seal is filled almost to the lower rin of the conical dome of the bell. The inner shell of the spirometer is constructed in such a manner that when the spirometer is empty the cone at the top of the inner shell fits into the dome of the bell.

In the Benedict-Roth and Krogh (fig. 21 and 22) apparatuses for the determination of oxygen consumption by volume change alone, without analyses of the respired air, minimizing the dead space of the apparatus is not required.

COUNTERPOISING THE SPIROMETER BELL

Accurate balance of the weight of the rising spirometer bell must be obobtained at all levels. The Krogh or bellows spirometer (fig. 22) is balanced by a counterpoise lever equipped with a weight which can be adjusted by altering its distance from the fulcrum. In the bell type of spirometer (figs. 19 and 21) the bell is usually suspended by a wire or string, which is attached to the center of the dome. Thence it passes up over a pulley to a counterweight which balances the weight of the bell.

A *small spirometer,* such as the Krogh (fig. 22) or Roth-Benedict (fig. 21), of not more than 10 liters capacity, can be made with such a light bell that pressure differences at various levels are negligible if the bell is balanced in mid-position by means of a counterpoise. One method of adjusting the counterpoise is to connect the spirometer, in the mid-position, with the outside air and adjust the weight of the counterpoise until the bell remains motionless. It is preferable, however, to adjust the weight with the aid of a water manometer connected with the air in the spirometer. The inertia and frictional resistance of the bell may be tested by moving it with the connecting tubing closed and noting by means of the water manometer the pressure when it comes to rest. The thermometer should be in place in the bell when the latter is balanced.

With *large spirometers,* such as the Tissot, some device must be employed to compensate at different levels for the increasing buoyant effect exerted by the water on the bell as the latter sinks into the fluid. This effect makes the bell require a heavier counterpoise to balance it when it is out of the water than when it is immerised. The devices commonly employed for increasing the pull of the counterpoise as the bell rises are diagrammatically illustrated in figure 19.

Figure 19, I, shows a cross section of one side of the spirometer bell, *2,* and its water seal, *1.* When the bell is immersed the water surface is at the

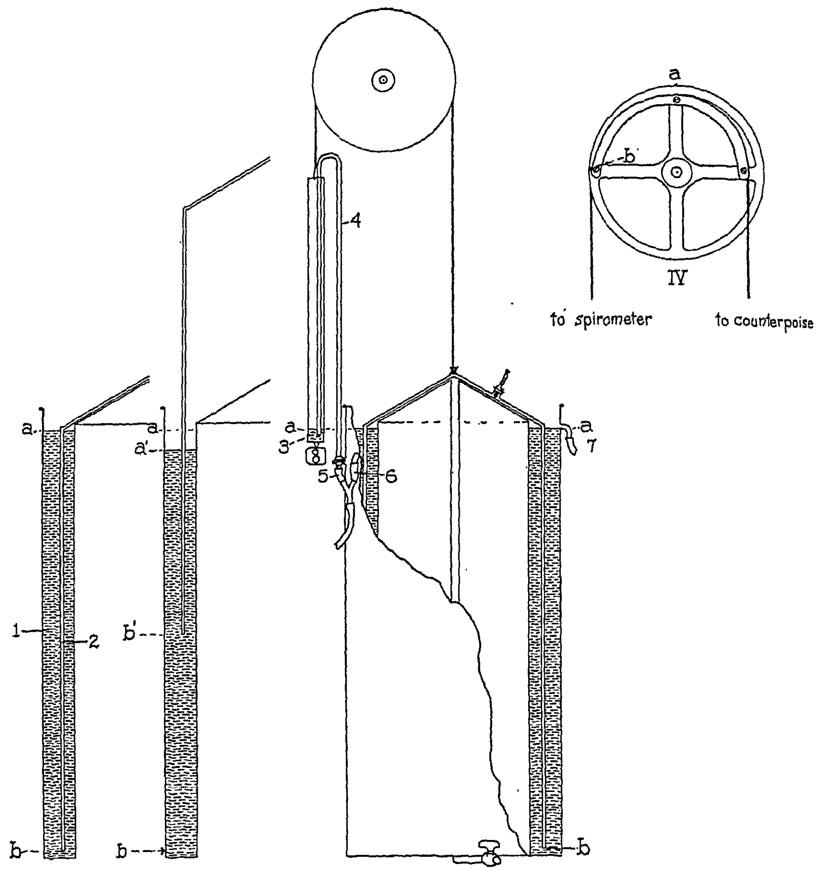


FIG. 19. General construction of Tissot bell spirometer and devices employed for automatic balancing of spirometer bells.

I. Cross section of one-half of spirometer, empty, showing the water seal *1*, the side wall of the bell *2* and the inner shell. The water is at the level *a*, almost, but not quite reaching to the dome of the apparatus. The length *ab* of the shell is immersed.

II. The spirometer has been partially filled with air so that the lower edge of the bell wall has risen from *b* to *b'*. The water level has consequently fallen from *a* to *a'* to fill the volume formerly occupied by the length *bb'* of the side wall of the bell. The apparent weight of the spirometer bell has increased by the weight of water equal in volume to the metal that has emerged. The fall in water level from *a* to *a'* does not occur when there is a device, like that shown in III, *7*, to keep the water level constant.

III. The general construction of a bell spirometer and an automatic compensating

initial level, a, the lower edge of the bell at level b. Therefore the length ab of the bell is immersed and displaces a volume of water equal to the volume of immersed metal. In accordance with the law of Archimedes the pull on the wire is diminished by the weight of the displaced water. In II the bell has risen by the distance bb', and the level of the water has fallen by the distance aa'. The walls of the bell have therefore emerged from the water by the distance $bb' + aa'$, and the pull of the bell on the supporting wire has been increased by loss of the buoyant effect of water on the metal formerly immersed.

Tissot's syphon compensating counterpoise. In order to compensate for the increase in weight of the emerging bell, Tissot (40) used a counterpoise, the weight of which was increased automatically by water siphoned into it as the bell rose. This device is diagrammatically represented in figure 19, III. The counterpoise consists of two parts, a lead weight *8* and a hollow metal cylinder *3*. This cylinder moves over a rigid syphon tube *4* which is connected with the water seal of the spirometer by means of two arms of a Y-tube (fig. 19, III, 5 and 6). The third arm of the Y is connected with a reservoir which is so adjusted that the water in the seal is maintained always at the same level, a. This adjustment is aided by means of the overflow tube *7*. The counterpoise is so adjusted that when the spirometer is empty and the water levels in both water seal and counterpoise tube are at a, the bell is exactly balanced. As the bell rises and the counterpoise cylinder falls, water passes into the latter from the water seal, the surface levels in both remaining constantly at a. The counterpoise cylinder is of such diameter that the weight of the water which it receives when the bell rises just balances the increase in weight of the emerging bell.

---

counterpoise. The counterpoise consists of two parts, a simple weight *8* and a hollow tube *3*. By means of a rigid syphon tube *4* water is transferred between the counterpoise tube *3* and the water seal of the spirometer in such a manner that the water level in the two is always the same. Therefore, when the bell rises water passes into the counterpoise to balance the apparent increase in weight of the bell. By the arm *5* of the Y tube the spirometer can be filled from a water reservoir which is also set, when the spirometer is in use, in such a manner that water enters the water seal through 6, to maintain the water level constant at a. *7* is an overflow tube which prevents the water level from rising above a.

IV. Another automatic compensating device. The spirometer is suspended by a wire which is attached at point a and moves over the periphery of a circular wheel. The counterpoise is attached at point b by another wire which moves over a spiral arc so constructed that, as the spirometer bell rises, the point at which the counterweight exerts its pull moves from a point nearer the center to one nearer the periphery of the spirometer pulley.

The area of the narrow circle which forms the inner cross section of the hollow counterpoise cylinder of figure 19, *3*, must equal the area of the thin ring of metal that is represented by a horizontal cross section of the spirometer bell. Then every centimeter that the bell rises out of the water will cause to siphon into the counterpoise a volume of water which just equals the volume of metal that emerges. The weight of water thus added to the counterpoise therefore balances the loss of buoyancy of the bell.

The necessary diameter of the counterpoise cylinder can be estimated either, 1, from the thickness and circumference of the metal wall of the bell, or 2, from the increase in weight shown by the bell as it emerges from the water.

1. To estimate from measurements the area of metal cross section of the bell, one measures in centimeters the circumference, C, and, with calipers, the thickness, b. With sufficient accuracy the area, A, is then calculated as the length, C, of the metal strip times its thickness, b, in cm.

$$A = C \times b \text{ cm.}^2$$

One then estimates the diameter, D, of a circle of equal area by the formula

$$D = 2\sqrt{\frac{A}{\pi}} = 1.127\sqrt{A}$$

Example. If the circumference, C, of a bell is 150 cm. and the thickness of the metal wall is 0.1 cm., $A = 150 \times 0.1 = 15$ cm.^{2}, and

$$D = 1.127 \times \sqrt{15} = 4.38 \text{ cm.}$$

The diameter, D, of the counterpoise cylinder which receives the syphoned water should, for this bell, therefore be 4.38 cm.

Theoretically a more precise calculation would be made by estimating A by the formula for calculating the area of a ring, $A = \pi(R_1^2 - R_2^2)$, where R_1 and R_2 are the outside and inside radii of the ring, which in this case is a cross section of the metal cylinder of the bell. This calculation gives slightly smaller A and D values, but the difference, with a thin, sheet metal bell, is not significant.

2. To estimate the diameter of the counterpoise cylinder from the determined increase in weight which the bell gains when it rises from the water one proceeds as follows. At first the bell is pressed down to its lowest position and while there is exactly counterpoised by a pail of shot, sand, or other adjustable mass at the end of the suspending wire, which is passed over a circular pulley. Then the bell is raised to its highest position, and brass

weights are added to the counterpoise sufficient to make the balance again perfect. The added weight, W, in grams, is used as follows to calculate the inner diameter, D, of the counterpoise cylinder which is to receive the syphoned water. From Archimedes' law,

$$W = H \times A$$

where H is the rise of the bell in centimeters and A is the cross section of bell metal in square centimeters. Hence

$$A = \frac{W}{H}.$$

From A thus found D is calculated as in the example given above. Accurate estimation of D will probably be found easier by this method than by method 1.

Compensating counterpoise on a spiral pulley. A somewhat simpler balancing device, which is now more generally employed, involves hanging the counterpoise from an eccentric pulley illustrated in figure 19, IV. The wire suspending the bell is attached at one point, a, on a circular pulley, while the wire supporting the counterweight, attached at another point, b, passes over a *spiral* arc on the side of the wheel. This arc is so arranged that, as the spirometer rises and its weight increases, the point at which the counterpull is exerted moves farther from the center. The spiral arc is so constructed that the change of leverage increases the pull of the counterpoise and exactly compensates for the increase in weight of the emerging spirometer bell.

The dimensions of the spiral arc can be calculated as follows. According to the law of levers, as the weight of the emerging bell increases, the spiral's radius, to the point, from which falls the wire supporting the counterpoise, must increase in the same ratio in order to maintain exact balance. If R_b is the radius of the spiral, to the point from which the wire falls to the counterpoise when the bell is at the bottom of the spirometer, and R_t is the corresponding radius when the bell is at the top, while W_b is the weight of counterpoise balancing the bell when the latter is at the bottom, we have, from the law of levers,

$$R_t : R_b = (W_b + H\ A) : W_b$$

CALIBRATION OF SPIROMETERS

A. Calibration by admission of measured volumes of air

This method can be applied to spirometers of either the Krogh or Tissot type. Successive known volumes of air are introduced and the rise of the

indicator point on the spirometer scale is noted. From the results the value of the constant K in the formula.

$$\text{Liters gas} = K \times \text{(millimeters rise of bell)}$$

can be computed. If the cross section of the bell is not uniform at all levels the fact will be revealed by variations in K, and an empirical curve can be drawn indicating the gas volumes corresponding to different scale readings.

The introduction of known volumes of gas can be effected with ease by means of the apparatus illustrated in figure 20, V. This consists of an aspirator bottle the lower opening of which is connected by rubber tubing with a large graduated leveling bulb or burette. In the upper opening is inserted, through a rubber stopper, a three-way stop-cock and a thermometer. One of the free ends of the stop-cock is connected with the spirometer, the other opens into the air of the room. Enough water is introduced into the bottle to cover the lower opening when the leveling bulb is full. The spirometer bell is lowered to the zero point. With the bottle stop-cock open to the room air the leveling bulb is filled to the desired mark, with the water in bottle and bulb at the same level. The stop-cock is then turned to connect the bottle with the spirometer, the desired volume of air is introduced by allowing that volume of water to pass from bulb to bottle. Water levels are in both vessels equalized, and the stop-cock is closed. The spirometer scale and the temperature of the air in the spirometer and that in the bottle are now read. The operation is repeated until the spirometer is filled.

If the temperature in bottle and spirometer are the same the spirometer volume at any point is the simple sum of the volumes measured from the bottle. If the temperature of the spirometer differs from that in the bottle a correction must be introduced, which may be calculated by means of the following equation:

$$V_2 = \frac{V_1\,(B - W_1)\,T_2}{(B - W_2)\,T_1}$$

Where V_1 is the volume of gas measured in the bottle at $(B - W_1)$ and T_1, V_2 is the same volume of gas after it has entered the spirometer where the temperature is T_2 and the pressure $B - W_2$. For values of W see table 18, page 163. In both bottle and spirometer the gas, because it is in contact with water, is saturated with water vapor.

Another means for introducing measured portions of gas is a pump[2] (see fig. 20, IV) devised by Benedict (11). It permits rapid calibra-

[2] This pump can be secured from W. E. Collins, 584 Huntington Avenue, Boston, Mass.

tion of large spirometers with considerable accuracy. It is built on the principle of an automobile grease gun and equipped with a three-way stop-cock. The pump is so constructed that it is air tight; the piston rod is prevented from twisting or turning in the barrel by the introduction of a key which is soldered to the top of the barrel and works in a slot in one side of the piston rod. The length of stroke is fixed by establishing metal to metal contacts at both ends of the stroke. When the handle is driven home it comes in contact with the key; when it is drawn out to its fullest extent a metal collar near the lower end of the rod strikes against the inside of the top of the pump. The pump is calibrated by discharging a definite number of strokes into an apparatus similar to that illustrated in figure 20, V, and measuring the amount of water displaced. For this purpose and for calibrating spirometers the air is drawn in over water through one opening of the pump stop-cock and delivered through the other into the spirometer or bottle. The pump must be absolutely air tight. The simplest way to test it is to produce slight pressure inside the pump and to note on a water manometer whether the pressure is sustained.

B. Calibration by measurement of cylindrical bell

If the bell is of circular cross section and of constant bore from top to bottom, it can be calibrated merely by measuring the circumference with a tape, and the thickness of the wall with calipers.

From the circumference, C one calculates the outside radius from the center of the bell to the outside of its wall, as

$$R_0 = \frac{C}{2\pi}$$

The radius of the cylindrical space inside the bell is

$$R_1 = R_0 - (\text{thickness of wall}).$$

Hence when both C and R are expressed in centimeters the area of the inner cylinder in square centimeters is:

$$\text{Area} = \pi R_1^2$$

and

$$V = H \times \text{Area} = H \pi R_1^2$$

where V is the volume in cubic centimeters of gas which the spirometer admits and H is the corresponding height in centimeters that the bell rises. By

this method one can calculate the volume change equivalent to a given change in the height of the bell. The value πR_1^2 is the apparatus constant, by which any reading of H is multiplied to obtain V. This method is advocated for the calibration of the Tissot spirometer by Boothby and Sandiford (17).

ESTIMATION OF INSTRUMENTAL DEAD SPACE

It is often necessary to know accurately the gas volume held by connecting tubes and other immovable parts of respiratory apparatus which can not be measured directly by the volumetric methods described above. The volume capacity of tubing can often be estimated with sufficient accuracy by calculation from measurements of bore and length. For parts of irregular contour the most practical method is the admixture of a known volume of some gas such as CO_2 or H_2, with the air contained in the dead space. The measured gas is caused to mix with air throughout the connecting tube or other parts of which the volume is sought, and a sample of the mixture is analyzed for the gas which was introduced. From the concentration of this gas the volume of the instrumental dead space may be computed by the formula:

$$V_D = V_G \times \frac{100 - \text{per cent } G}{\text{per cent } G}$$

where V_D is the volume of the dead space, V_G the volume of gas (CO_2 or H_2) mixed with the air in the dead space, and per cent G is the per cent of the gas (CO_2 or H_2) found in the final mixture of gas and dead space air.

ALCOHOL CHECKS FOR THE CONTROL OF METABOLISM APPARATUS

No amount of care taken in the manufacture, calibration and manipulation of the apparatus can give the sense of assurance that comes from an accurate control determination of the oxygen consumed in the combustion of some known substance. For this reason "alcohol checks" have become the accepted criteria and are cited by the most careful observers as evidence of technical accuracy. They should be carried out before new apparatus is used, and whenever there is reason to believe that its accuracy requires confirmation.

An alcohol check is carried out by burning a known quantity of ethyl alcohol in a device connected with the respiratory apparatus. The O_2 consumed, or the CO_2 produced, or both, are determined in a manner similar to that followed when the apparatus is used to measure respiratory metabolism. In the alcohol check determination a special alcohol burner is substituted for the subject connected with the respiratory apparatus.

Benedict (11) has devised for the determination of alcohol checks an ingenious apparatus which can be adapted to any of the various types of instruments used in the measurement of respiratory metabolism. The apparatus is illustrated in detail, as assembled for use, in figure 20, I, II, and III. It consists of a calibrated microburette connected by a fine rubber tube to a length of capillary glass tubing in the upper end of which is inserted a fine asbestos wick. The capillary tube with its wick acts as a burner and is inserted in a glass lamp chimney which is set in a water seal below. Mica baffle plates above and below the burner protect it from drafts. The air or oxygen passes into the chimney from below and escapes above through a tube which passes through a rubber stopper in the top of the chimney. The current of air or oxygen is maintained by means of a small spirometer which serves as a pump, and is activated by the lever of a Ford wind-shield cleaner. The same motive power by means of ratchet gears gradually raises the alcohol burette which is suspended by a string. This keeps the alcohol feed constant throughout the determination. The wind shield cleaner is run by laboratory air pressure or vacuum, or by a suction pump.

In practice the alcohol check machine is attached in the place of the patient to the metabolism apparatus. The flame is lighted and burner, burette and the small spirometer which acts as an artificial lung are adjusted so that the flame burns smoothly and air current and oxygen consumption proceed at a rate approximating that of a usual metabolism determination. When adjustment is complete the metabolism apparatus is set as for a regular determination and run for a period of time measured by a stop-watch. Burette readings are made at the beginning and at the end of the run, to measure the volume of alcohol burned.

$$C_2H_5OH + 3\ O_2 = 2\ CO_2 + 3\ H_2O$$

$$\frac{CO_2}{O_2} = R.\ Q. = \tfrac{2}{3} = 0.67.$$

The molecular weight of alcohol is 46. Therefore, for 1 mole (46 grams) of alcohol, 3 moles of O_2 ($3 \times 22.4 = 67.2$ liters of O_2) will be consumed and 2 moles of CO_2 ($2 \times 22.4 = 44.8$ liters of CO_2) will be produced. Or the combustion of 1 gram of alcohol involves the consumption of

$$\frac{67.2}{46} = 1.46 \text{ liters of } O_2.$$

and the production of

$$\frac{44.8}{46} = 0.974 \text{ liters of } CO_2,$$

The exact strength of the alcohol must be determined by measurement of specific gravity or refractive index with the aid of standard tables. From

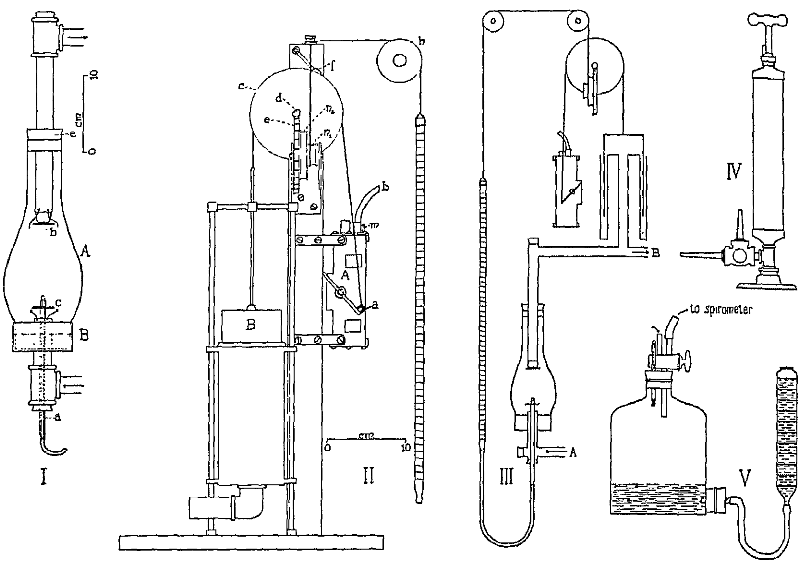


FIG. 20. Alcohol check apparatus of Benedict (10) and devices for calibrating spirometers (11).

I. Burner. *A*, glass lamp chimney in water seal in brass cup. *B*, *a*, glass burner; *c*, mica disk, and *b*, baffle plate for protection of flame; *e*, rubber stopper.

II. Wind-shield cleaner, artificial lung and burette. *A*, wind-shield wiper with rocker arm, *a*; *b*, rubber tubing connecting with vacuum or compressed air line; *m*, screw to control motion of wind-shield wiper; *h* and *c*, pulleys; *B*, bell of spirometer acting as counterweight; *d* and *e*, worm gears actuating wooden spools, n_1 and n_2; *f*, pawl permitting movement of pulley, *c*, in only one direction.

III. Alcohol check apparatus of Benedict assembled.

IV. Benedict (11) pump for calibration of respiratory apparatus.

V. Simple device for calibration of spirometer.

these data the volume of alcohol burned during the run can be converted into terms of grams of C_2H_5OH (see table 10 in chapter I).

The observed oxygen consumption and CO_2 production should not differ from theoretical by more than 1 per cent in a satisfactory test.

BASAL METABOLISM

In the chapter on total metabolism in volume I it has been shown that the heat production and the nature of the fuel consumed by the body can be estimated by measuring the oxygen consumption, the carbon dioxide production, and the nitrogen excretion, the last serving as a measure of the protein burned. The theoretical considerations on which this conclusion are based have been discussed in that chapter.

Possibility of estimating basal metabolism from only oxygen intake or carbon dioxide output

For the determination of total metabolism under varied conditions only apparatus which permits determination of both oxygen consumption and carbon dioxide production is exact. The number of calories produced per liter of O_2 consumed or CO_2 produced varies with the respiratory quotient, as shown in table 19. Unless both CO_2 output and O_2 intake are known one therefore does not know by what factor to multiply the liters of O_2 consumed or CO_2 excreted in order to estimate the calories produced. However, in the resting, post-absorptive state used for basal metabolism observations, most individuals except those with severe diabetes burn much the same food mixture, yielding respiratory quotients which are relatively constant between 0.80 and 0.85. Under these circumstances it may be assumed that the respiratory quotient, and consequently the caloric values of oxygen and of carbon dioxide, are fairly constant.

Under the conditions of basal metabolic rate determinations therefore, either O_2 intake or CO_2 output alone can be used as an approximate measure of the heat production. The use of O_2 intake is, however, subject to presumably less error than that of CO_2 output, for the following two reasons:

1. The caloric value of a liter of O_2 consumed varies, for a given change in R. Q., only about one-fourth as much as the caloric value of a liter of CO_2 produced. Thus from table 19 one sees that changing R. Q. from 0.80 to 0.85 changes the caloric value of O_2 only from 4.801 to 4.862, or 1.3 per cent, while the caloric value of CO_2 changes from 6.002 to 5.722, or 4.7 per cent.

2. Because of the large reservoir of loosely bound CO_2 in the body, any acceleration of breathing results in a temporary increase in the rate of CO_2 output, due to pumping CO_2 out of the reservoir and not to increased CO_2 formation. Later there occurs a compensatory period of retarded CO_2 output during which the reservoir is refilled. Changes in respiratory rate have much less effect upon O_2 intake, since the only important reservoir of

loosely bound O_2 is the relatively small one of the blood hemoglobin. This is normally 95 per cent saturated in the lungs, and acceleration of breathing can not make the arterial blood take up appreciably more oxygen.

For these reasons all routine clinical procedures in general use for basal metabolic rate determination by a single gas measure the O_2 consumption rather than the CO_2 output.

The rate of CO_2 output can, however, be measured very simply, and King (31) has collected data, from observations in which both CO_2 and O_2 were both determined, indicating that, under the conditions of basal metabolic rate measurement in non-diabetic subjects, CO_2 output may be as accurate a measure of metabolism as O_2 intake. It may be that CO_2 production has been unjustly neglected for basal metabolic rates, and that the possible errors above discussed are not actually important under the special conditions employed.[3]

Preparation of subject for basal metabolism determination

The factors which affect respiratory metabolism in general have been detailed in the chapter on total metabolism in volume I. Only those involved in the preparation for basal metabolism determination will be described here.

Food. It is generally held that no food should be eaten for at least 14 hours prior to the determination of basal metabolism. Benedict and Benedict (6) have, it is true, shown that a small meal containing no ketose sugars

[3] King (32) states that the CO_2 method gives satisfactory results in basal metabolic rates except in severe diabetes, where the respiratory quotient is below 0.80 and may be near 0.70. With regard to the effect of variable breathing rate he states: "It is well known that a nervous subject may superventilate the lungs to such a degree, that carbon dioxide is washed out of the blood in excess of the amount that is being formed in the system during the test. In such a case there is an excessive elimination of the gas for the first period of the test, whether it be ten or fifteen minutes or less. I have found, however, that the available excess of carbon dioxide in the blood is washed out under such conditions in a short time, the exact time depending upon the degree of superventilation. If a second period be carried out it is found that there is a marked falling off in the carbon dioxide output as compared with the first period, even though superventilation be persisted in. For this reason two periods are always advisable as against one in using the carbon-dioxide collection method. If results for the two periods agree within 10 per cent, the effects of possible superventilation are negligible. If a difference exceeding 10 per cent is obtained a third determination practically always shows a reading about half way between the first two readings. This is due to the tendency of the body to store during the second period an amount of carbon dioxide to compensate for the amount that was washed out in the first period, for usually the superventilation ceases after a comparatively few minutes, owing to the induction of a tendency to apnea."

and minimal amounts of protein does not produce any measurable change of the metabolism of normal individuals. The meal which they propose consists of 200 cc. of caffeine-free coffee sweetened with saccharine, 30 grams of medium cream and 25 grams of potato chips. As they point out, it is not safe to conclude that such a meal, because it does not influence the metabolism of normal persons, will be equally without effect on patients with diseases of various kinds. For this reason it is safer to adhere to the generally accepted rule permitting no food to be taken between the evening meal of the preceding day and the time of the metabolism determination. Other permissible breakfasts have been proposed (5, 39). Stimulants and drugs should be avoided. Krogh (34) believes that a more accurate measure of the basal metabolism is secured if the subject subsists for one or more days preceding the test on a low-protein, high-carbohydrate diet. To introduce such a preliminary period as part of the routine clinical procedure is, however, not generally practicable.

Rest. The subject should be completely at rest and in a recumbent or reclining position during the test. The ideal condition is to make the test before the subject has arisen in the morning. When this is impossible the subject should rest in the reclining or recumbent position for at least thirty minutes before the determination is begun. One-half hour gives time enough for the effects of the usual walk to the place of examination to disappear in normal individuals (15). In patients a longer period is often necessary, especially if they are suffering from diseases which affect the circulatory or respiratory system. Both pulse and respirations should be allowed to reach a constant minimal rate before the determination is begun. However, too long a delay may cause nervousness and restlessness.

Mental quiet is as important as physical rest. During the preliminary period the subject should be protected from all environmental stimuli and causes for anxiety. It is sometimes necessary to emphasize the harmless nature of the test itself. Although complete physical and mental repose is desirable, the subject should not be allowed to sleep during the determination, because the metabolism during sleep is 8 or 9 per cent lower than during the waking basal state.

Practice tests. Few untrained subjects, when a mouth-piece or mask is first applied, fail to exhibit abnormalities of respiration. Usually these abnormalities are transient, disappearing in a short time as the subject appreciates that there is no danger of smothering and no interference with respiration. Occasionally the initial disturbance is so great and prolonged that it is impossible to secure an acceptable determination on the first day. In any case it is well to go through all the motions of a test in order to

accustom the subject to the procedure so that succeeding measurements may be undisturbed.

When a mouth-piece is to be used this is first applied and adjusted to the comfort of the patient. When he has become accustomed to the mouth-piece, the nose-clip is applied. While the observer is testing for and excluding leaks about the mouth and nose a further opportunity is given for the subject to become used to the procedure. He is then connected with the apparatus. It is usually well, by means of a cut-off valve, to permit him to breathe room air for a short period before commencing the determination. Finally, when the respirations are quiet and perfectly regular, the valve is turned and the determination begun.

In no case should the result of a single determination be accepted.

Control of activity. A record must be kept of activity during the test. All movements of the subject must be noted. Any undue restlessness is cause for discarding the results of a determination. Such restlessness is less likely to occur if the utmost attention is given to the comfort of the subject. Variability of the pulse or respirations or any striking change in the character or rate of either during the course of the test casts suspicion upon its validity.

Subjective and objective errors. Objective sources of error, proceeding from leaks or other faults in apparatus or analytical technique, give values that may be either too high or too low. Subjective sources of error, proceeding from abnormal respirations, insufficient rest, restlessness or nervousness, are more likely to yield high than low values. If, therefore, the results of duplicate determinations differ by more than 5 per cent and technical errors can be excluded, it is preferable to accept the lower result rather than the higher or an average of the two. In such circumstances, however, the determination should be repeated before any definite conclusions are drawn.

Graphic recording devices facilitate the detection of respiratory abnormalities and the respiratory record should be given due weight in the evaluation of the results of any test. Basal respiratory quotients greater than 1.00 or less than 0.71 indicate overventilation or abnormally restricted breathing respectively, and can not be used in calculation of oxygen consumption.

DETERMINATION OF OXYGEN CONSUMPTION AND CARBON DIOXIDE PRODUCTION WITH THE TISSOT (40) OPEN CIRCUIT APPARATUS (2, 17, 22)

Principle

The subject through a mouth-piece or mask equipped with three-way valves inspires outdoor air, which is preferable to ordinary room air because

of more constant composition, and exhales into the spirometer. The expired air is collected in the spirometer for a known period of time. A sample, taken from the spirometer, is then analyzed. From its volume and CO_2, O_2, and N_2 content, compared with the composition of the outdoor air inspired, the consumption of O_2 and production of CO_2 can be calculated.

The spirometer used, of the type shown in figure 19, should be capable of receiving at least 80 liters of air, in order to make possible the use of periods not shorter than ten minutes.

Details of procedure

Before the test the spirometer is emptied. The subject is prepared in the usual manner for metabolism determinations. The nose-clip and mouth-piece, or the mask with its air-valves attached, is applied and connected with the source of inspired air and with the apparatus. The latter should be equipped with a three-way cut off valve near the subject, which diverts the expired air from the spirometer into the room until the actual determination is begun. When the subject is comfortably adjusted and breathing naturally this valve is turned to direct the expired air into the spirometer. After a few liters have been collected, the valve is again turned and the collected air is expelled from the machine. This procedure is repeated two or three times. It serves a double purpose: it accustoms the subject to breathing into the spirometer, and it washes the atmospheric air out of the instrumental dead space with expiratory air.

When the dead space has been thus washed out the spirometer is again set at the zero mark. The operator, with a stop-watch in one hand and the inlet valve in the other, waits until the subject is breathing quietly. At the end of an inspiration he turns the spirometer cock to divert the expired air into the spirometer, at the same time starting the stop-watch. After approximately ten minutes, or when the spirometer is almost filled to its practical capacity, at the end of another inspiration the valve is turned back and the stop-watch is stopped simultaneously.

Readings are made of the volume and temperature of the gas in the spirometer. A sample of the gas is then withdrawn in a sampling bulb. (The cock from which air samples are withdrawn from the spirometer is sometimes placed on the inlet tube, preferably on the bell.) Before a sample is taken some of the gas in the spirometer is wasted to wash out connecting tubes. After the first sample has been withdrawn, and before the duplicate sample is taken, further gas may be wasted and the second sample drawn from the half emptied spirometer, to insure against

errors from layering in the spirometer. If, however, the subject has breathed normally and regularly throughout the test, the composition of the gas mixture should be so uniform that errors from layering are impossible (24).

The spirometer is now emptied completely in preparation for the duplicate test, which is carried out like the first.

The gas mixture is not allowed to stand in the spirometer longer than necessary before sampling, because slow diffusion, especially of CO_2, takes place between the gas mixture and atmospheric air through the water seal.

The samples are analyzed for CO_2, O_2, and N_2, as described in the chapter on analysis of gas mixtures.

Calculation of results from Tissot apparatus

The *volume of expired air* observed in the spirometer is reduced to 0°, 760 mm. by multiplication with the factor

$$\frac{B - W}{760\ (1 + 0.00367\ t)},$$

for which values are given in table 15 on page 129 of chapter III. Representing the observed volume of expired air in the spirometer as $V_{obs.}$, and the volume reduced to 0°, 760 mm. as $V_{air\ exp.}$

$$V_{air\ exp.} = V_{obs.} \times \frac{B - W}{760\ (1 + 0.00367\ t)}.$$

The *volume of inspired air* is usually somewhat greater than that of the expired air measured in the spirometer. The volume change is due to the fact that whenever the R. Q. is less than 1.00, as is usually the case, the O_2 removed from the air in the lungs is only partly replaced by CO_2. In consequence the total air volume shrinks, and the N_2 content increases proportionally. Hence the change in N_2 percentage from the value 79.04, which it has in atmospheric air, enables one to calculate the volume of inspired air from that observed of the expired.

$$V_{air\ insp.} = V_{air\ exp.} \times \frac{\text{Per cent } N_2 \text{ in expired air}}{79.04}$$

$$V_{O_2\ insp.} = \frac{\text{Per cent } O_2 \text{ in inspired air}}{100} \times V_{air\ insp.}$$

The oxygen content in inspired air is 20.93 per cent, except, as stated before, in winter in a place as congested as New York. For ordinary conditions one can therefore calculate:

$$V_{O_2\ insp.} = \frac{20.93}{100} \times V_{air\ insp.}$$

$$V_{O_2\ exp.} = \frac{\text{Per cent } O_2 \text{ in expired air}}{100} \times V_{air\ exp.}$$

$$O_2\ consumed = V_{O_2\ insp.} - V_{O_2\ exp.}$$

Similarly

$$V_{CO_2\ insp.} = \frac{\text{Per cent } CO_2 \text{ in outside air}}{100} \times V_{air\ insp.}$$

$$= \frac{0.03}{100} \times V_{air\ insp.}, \text{ for ordinary fresh air.}$$

$$V_{CO_2\ exp.} = \frac{\text{Per cent } CO_2 \text{ in expired air}}{100} \times V_{air\ exp.}$$

$$CO_2 \text{ produced} = V_{CO_2\ exp.} - V_{CO_2\ insp.}$$

$$\frac{CO_2 \text{ produced}}{O_2 \text{ consumed}} = \text{respiratory quotient, R. Q.}$$

The calculation of the calories produced per hour is discussed below.

USE OF CLOSED-CIRCUIT APPARATUS FOR THE DETERMINATION OF OXYGEN CONSUMPTION ONLY[4]

Details of apparatus

The general principles of the apparatus are illustrated in figure 21, a schematic diagram of the *Roth-Benedict* (*38*) *apparatus*. It consists essentially of a circuit of tubes in which the air current is directed by means of two flutter-valves. The expired air passes, by way of the inlet tube, through an absorber containing soda lime, into a spirometer in which it becomes

[4] The most popular machines of this type are the Roth-Benedict (38) made by Warren E. Collins, Boston, Mass.; the Sandborn Graphic, made by Sandborn, 1048 Commonwealth Avenue, Boston, Mass.; and the Krogh (33, 34) sold by H. N. Elmer, Chicago, Ill. All these are equipped with graphic recording devices and all depend on valves for the maintenance of circulation. Sandborn has recently advertised a graphic machine in which the circulation is maintained by means of a fan.

mixed with oxygen. It then passes out through an opening in the bottom of the spirometer to the outlet tube which conducts it back to the subject. The spirometer is carefully balanced and calibrated. The volume of gas can be read from the millimeter scale, *e*, on the right by means of a pointer attached to the counter weight *d*. To the same weight is attached the pen which writes on the kymograph drum. The spirometer bell has a volume of about 8 liters.

The three-way valve, *a* interposed between the subject and the apparatus, although not regularly included in the apparatus, is a convenient addition. It can be used to divert the respiration to the room air before and after a determination, or momentarily during a determination, without removing the mouth-piece. It is convenient in testing for leaks.

Through stop-cock *b* oxygen can be introduced. The same stop-cock permits the withdrawal of samples of air for analysis.

Stop-cock *c* is used to drain the water from the spirometer when the water in the seal needs to be changed.

A themometer *f* inserted in the top of the bell, indicates the temperature of the gas.

Testing the Apparatus. **The calibration of the apparatus should be carefully verified by the usual methods used for the calibration of spirometers. To test for leaks, valve *a* is closed, or the mouth-piece tube is closed with a rubber stopper, the temperature and volume of the gas in the spirometer are read, and a weight of about 100 grams is placed on the top of the spirometer. After a period of fifteen to thirty minutes the weight is removed and the temperature and volume of gas are again read. The volume will diminish if the apparatus leaks, (allowance must, of course, be made for temperature changes). If the gas mixture in the apparatus contains appreciable amounts of carbon dioxide this will be slowly absorbed by the water in the water seal during the test with a resultant diminution of gas volume that will simulate a slight leak. Such CO_2 absorption differs from a leak, however, in the fact that it is not continuous, but rapidly self-terminative.**

Soda-lime. The soda lime used in the absorber must be finely enough subdivided to afford a large absorbing surface, but not so fine that it offers undue resistance to the passage of air. It must also be of such a quality that it does not easily become caked. The most satisfactory results have been secured by using 4 to 8 mesh "Wilson" (41) soda-lime, which has a high moisture (15 to 19 per cent) and low alkali (5 per cent) content. Roth (38) states that 3 pounds of soda lime in the Roth-Benedict apparatus will suffice

for 60 to 70 ten-minute tests. If the efficiency of the absorber is in doubt it can be tested in one of two ways:

1. Immediately after a test run valve *a* is closed. The spirometer is then alternately raised and lowered a slight distance by hand (not so far that the water from the seal is splashed into the soda lime or room air permitted to enter the apparatus) to mix the air in the spirometer and to drive it slowly

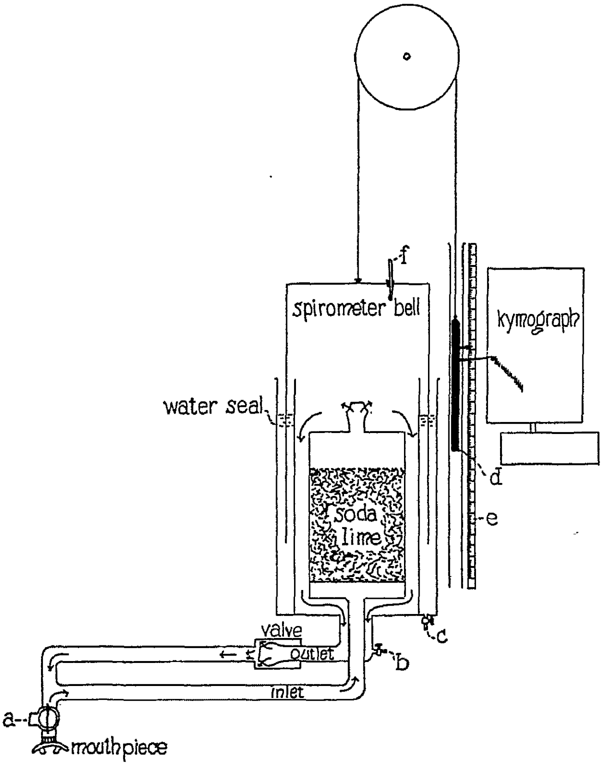


FIG. 21. Diagrammatic representation of the Roth-Benedict apparatus for the determination of basal metabolism.

around the circuit. If the soda lime is not efficient the gas in the spirometer will contain an appreciable amount of CO_2 which will be absorbed gradually, causing the volume of gas to diminish.

2. At the end of a test a sample of gas may be taken through the stop-cock *b* and analyzed for carbon dioxide.

If the soda lime is grossly inefficient the volume of air breathed per minute

by the subject, and usually the depth of individual respirations, will increase steadily during the course of a determination because of the stimulating effect of the accumulating carbon dioxide. This rise in ventilation may never become great enough to cause subjective distress or to become apparent to the observer's eye, but is easily detected on a kymographic tracing.

Wilson (41) suggests examining the soda lime at frequent intervals and replacing only that portion of it which has become moist and caked. This is more economical than less frequent periodical replacement of all the soda lime.

Procedure

If a kymographic device is used enough oxygen is admitted to the spirometer from an oxygen tank (equipped with reducing valves) connected with stop-cock *b* to bring the writing point to a convenient level near the bottom of the paper. The kymograph and time recorder are started and the pens tested. The patient, after the usual preparatory procedures, is connected with the apparatus. During the run the bell oscillates with each respiratory cycle, producing on the kymograph a record of the individual respirations. As the oxygen is absorbed the bell gradually falls and the kymographic record rises. When the latter approaches the top of the paper fresh oxygen may be admitted and a new test begun at once. The temperature of the gas in the spirometer is read at the beginning and end of each determination by means of the thermometer *f*.

It is not well to carry out more than two determinations without an interruption. During such an interruption it is advisable to disconnect the subject from the apparatus to afford him a rest from the mouth-piece and nose-clip.

In a satisfactory determination the record on the kymograph should be regular and should ascend in an even slope. The slopes of the records of duplicate determinations should be equal.

If no recording device is used a slightly different technique is employed. In this case the test must be begun and ended with the subject in ***the same phase of respiration*** (as a rule, at the end of a normal expiration), to avoid changes between initial and final readings due to variations in the volume of gas in the patient's lungs. The spirometer is filled with oxygen in the usual manner. By means of valve *a* the patient, although connected with the apparatus, is at first permitted to breathe room air. The temperature and volume of the gas are read by means of

thermometer *f* and scale *e* respectively. At the very end of a normal expiration the observer turns valve *a* to connect the subject with the spirometer and at the same instant starts a stop-watch. When the volume of oxygen has diminished as far as is practicable, the valve is turned at the end of an expiration, and the stop-watch is stopped. The temperature and volume of the gas are again read.

Calculation

If a graphic device is used the kymographic paper is removed from the drum and spread on a flat table. With a straight edge a line is drawn along the lower border of the kymographic tracing (extreme expirations). If the

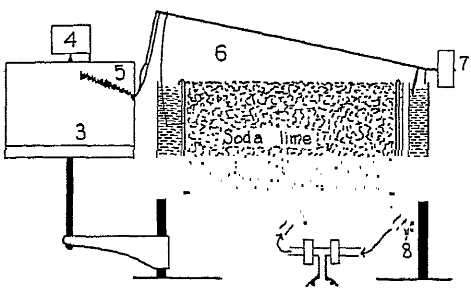


FIG. 22. Krogh's (33, 34) spirometer for the determination of basal metabolism. *1*, outlet tube; *2*, inlet tube, both connect with valved mouth-piece; *3*, kymographic drum *4*, clockwork motor to drive kymograph; *7*, counterpoise; *8*, pet-cock to drain off water of condensation or to permit sampling of residual gas. The soda lime container has a capacity of 8 liters.

test has been satisfactory the expiratory points should lie along a smooth line. The height by which this line rises above the base line in a given period is a measure of the oxygen consumed in that time. The mathematical conversion of this measurement to terms of volume of oxygen at standard conditions (0°, 760 mm.) depends upon the type of machine employed. If no graphic device is used the oxygen consumption is calculated from the difference between the readings on scale *e*, (fig. 21) before and after the test.

If, as is customary, it is assumed that the respiratory quotient is 0.82, each liter of consumed O_2, calculated to 0°, 760 mm. (see table 19, p. 206) represents 4.825 calories. If Krogh's (34) high carbohydrate diet has been given on preceding days a caloric value of 4.9 calories per liter of O_2 (R. Q. = 0.89) is used instead.

In apparatus like the Roth-Benedict, without a circulating pump, a small

amount of CO_2 usually remains unabsorbed for a time after the determination. However, the percentage of residual CO_2 remains relatively constant while the apparatus is in use; therefore, if a short preliminary run is made, the error from unabsorbed carbon dioxide is negligible because it affects equally the initial and final readings.

Krogh's (33, 34) *apparatus*, illustrated in figure 22, utilizes the same principles as the Roth-Benedict. It is, however, equipped with the bellows type of spirometer.

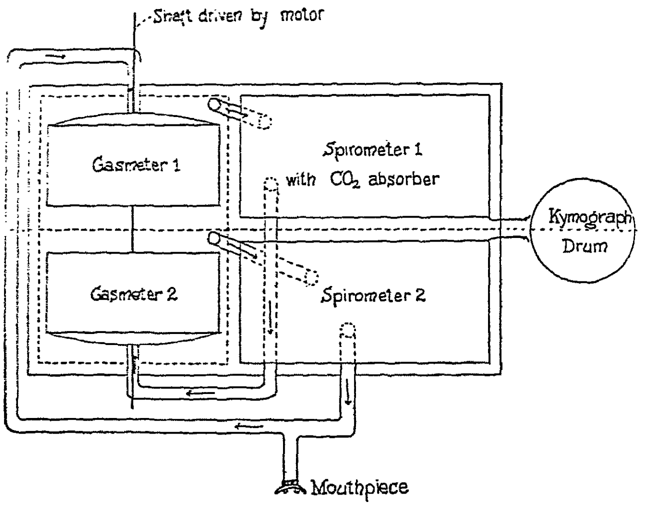


FIG. 23. Hagedorn's (28) closed circuit apparatus with graphic device for the determination of respiratory metabolism.

HAGEDORN (28) CLOSED-CIRCUIT APPARATUS WITH GRAPHIC DEVICE FOR RECORDING BOTH CO_2 AND O_2

Hagedorn (28) has described an ingenious form of closed circuit apparatus which provides a graphic record of respirations, CO_2 production, and O_2 consumption. The principle of the apparatus was first employed by Hanriot and Richet (29a). The latter authors originally used three gas meters of equal size. One measured the volume of the inspired air, another that of the expired air, and the third that of the expired air after its carbon dioxide had been removed. The difference between the second and third volumes represented carbon dioxide produced; the difference between the first and third represented oxygen consumed. Differences in the speed or

pressure in the gas meters introduced errors in gas measurements. To obviate this difficulty Hagedorn has connected in series two equal balanced spirometers of the Krogh type, one containing a soda-lime absorber for removal of CO_2, and two equal gas meters driven at a constant rate by a motor to which they are connected by a single shaft.

This apparatus is diagrammatically represented in figure 23. The meters are immersed in a common water bath in which the level of water can be adjusted so that it is the same in both meters. The two spirometers, equipped with recording pens, are connected in series with the gas meters in a closed circuit. The subject is connected with the circuit at one point. The motor-driven gas meters produce a constant circulation through the apparatus. The air passes through gas meter *1* to spirometer *1*, containing the soda-lime, then through gas meter *2* to spirometer *2* and back to the subject. The subject is connected with the circuit by a side-arm between spirometer *2* and gas meter *1*.

By means of a writing device the motions of both spirometers are recorded. As in other closed circuit machines, air enriched with oxygen is employed.

The gas meters and spirometers must be so exactly balanced that the pressure in all units remains the same throughout.

Carbon dioxide production and oxygen consumption are calculated from the changes in the volumes of gas in the two spirometers.

CALCULATION OF TOTAL METABOLISM

For the calculation of total metabolism one must know not only the amounts of oxygen consumed and carbon dioxide produced, but also the rate of protein catabolism.

From the data secured by means of respiratory metabolism apparatus the CO_2 production and O_2 consumption per hour are estimated by the formulae

$$(1) \qquad \frac{60\ (O_2 \text{ consumed})}{(\text{time of test in minutes})} \quad O_2 \text{ per hour.}$$

$$(2) \qquad \frac{60\ (CO_2 \text{ produced})}{(\text{time of test in minutes})} : \quad CO_2 \text{ per hour.}$$

The protein metabolism is calculated from the nitrogen excreted in a given period of time (usually one or two hours) including the period of the respiratory metabolism test.

$$(3) \qquad \frac{60\ (N \text{ in urine})}{(\text{time of urine collection in minutes})} = N \text{ per hour.}$$

TABLE 19

Analysis of the Oxidation of Mixtures of Carbohydrate and Fat (from Lusk (35))

R.Q.	Percentage of total oxygen consumed by		Percentage of total heat produced by		Calories per liter of	
	Carbohydrate (1)	Fat (2)	Carbohydrate (3)	Fat (4)	O_2 (5)	CO_2 (6)
0.707	0	100.0	0	100.0	4.686	6.629
0.71	1.02	99.0	1.10	98.9	4.690	6.605
0.72	4.44	95.6	4.76	95.2	4.702	6.533
0.73	7.85	92.2	8.40	91.6	4.714	6.459
0.74	11.3	88.7	12.0	88.0	4.727	6.388
0.75	14.7	85.3	15.6	84.4	4.739	6.320
0.76	18.1	81.9	19.2	80.8	4.751	6.252
0.77	21.5	78.5	22.8	77.2	4.764	6.186
0.78	24.9	75.1	26.3	73.7	4.776	6.122
0.79	28.3	71.7	29.9	70.1	4.788	6.062
0.80	31.7	68.3	33.4	66.6	4.801	6.002
0.81	35.2	64.8	36.9	63.1	4.813	5.942
0.82	38.6	61.4	40.3	59.7	4.825	5.883
0.83	42.0	58.0	43.8	56.2	4.838	5.829
0.84	45.4	54.6	47.2	52.8	4.850	5.775
0.85	48.8	51.2	50.7	49.3	4.862	5.722
0.86	52.2	47.8	54.1	45.9	4.875	5.668
0.87	55.6	44.4	57.5	42.5	4.887	5.616
0.88	59.0	41.0	60.8	39.2	4.899	5.566
0.89	62.5	37.5	64.2	35.8	4.911	5.518
0.90	65.9	34.1	67.5	32.5	4.924	5.471
0.91	69.3	30.7	70.8	29.2	4.936	5.423
0.92	72.7	27.3	74.1	25.9	4.948	5.378
0.93	76.1	23.9	77.4	22.6	4.961	5.333
0.94	79.5	20.5	80.7	19.3	4.973	5.288
0.95	82.9	17.1	84.0	16.0	4.985	5.243
0.96	86.3	13.7	87.2	12.8	4.998	5.202
0.97	89.8	10.2	90.4	9.58	5.010	5.163
0.98	93.2	6.83	93.6	6.37	5.022	5.124
0.99	96.6	3.41	96.8	3.18	5.035	5.085
1.00	100.0	0	100.0	0	5.047	5.047

Formulae by which values in numbered columns are calculated. The R.Q. is indicated as R.

$$\text{(1) Per cent} = 100\,\frac{R - 0.707}{0.293}$$

$$\text{(2) Per cent} = 100\,\frac{1.00 - R}{0.293}$$

$$\text{(3) Per cent} = \frac{504.7\ (R - 0.707)}{5.047\ (R - 0.707) + 4.686\ (100 - R)}$$

$$\text{(4) Per cent} = \frac{468.6\ (1.00 - R)}{5.047\ (R - 0.707) + 4.686\ (1.00 - R)}$$

$$\text{(5) Calories} = 4.686 + \frac{R - 0.707}{0.293} \times 0.361$$

$$\text{(6) Calories} = 5.047 + \frac{1.00 - R}{0.293} \times 1.582$$

In the combustion of the amount of protein which yields 1 gm. of urinary nitrogen 5.94 liters of O_2 are consumed and 4.76 liters of CO_2 are produced.

(4) Gm. N per hour $\times$ 5.94 = liters of O_2 per hour used in combustion of protein.

(5) Gm. N per hour $\times$ 4.76 = liters of CO_2 per hour produced by combustion of protein.

(6) (Total liters of O_2 consumed per hour) $-$ (N per hour $\times$ 5.94) $\doteq$ consumption of oxygen in combustion of carbohydrate and fat = liters of non-protein O_2 per hour.

(7) (Total liters of CO_2 produced per hour) $-$ (N per hour $\times$ 4.76) = liters non-protein CO_2 per hour.

(8)
$$\frac{\text{Liters of non-protein } CO_2 \text{ per hour}}{\text{Liters of non-protein } O_2 \text{ per hour}} = \text{non-protein R. Q.}$$

From the non-protein R. Q., by columns numbered 1, 2 and 3 of table 19, it is possible to calculate the relative amounts of fat and carbohydrate oxidized during the metabolism determination. The caloric equivalents of CO_2 and O_2, which vary with the relative proportions of fat and carbohydrate in the metabolic mixture, are given in columns numbered 5 and 6 of table 19.

(Liters of non-protein O_2 per hour) $\times$ (Calories per liter of O_2 corresponding to observed non-protein R. Q.) = non-protein calories per hour.

(Liters of O_2 per hour used in combustion of protein) $\times$ 4.485 = Calories per hour from combustion of protein. Hence (Gm. N per hour) $\times$ 26.6 = Calories per hour from combustion of protein.

Protein Calories + Non-protein Calories = Total Calories per hour.

In many experiments urinary nitrogen is not determined. In this case it is usually assumed (17) that the total calories are derived from the combustion of carbohydrate and fat. As the calories yielded per liter of oxygen in combustion of both these foods (5.047 and 4.686 calories, respectively) are more than in combustion of protein (4.485), the value obtained by this method of calculation is systematically somewhat high, although the error seldom exceeds 3 per cent of the total.

If only oxygen is determined it is usually assumed that all the calories were derived from carbohydrate and fat with an average R. Q. of 0.82. The calculation, therefore, is simplified to

(Liters of total O_2 consumed per hour) $\times$ 4.825 = Calories per hour.

CALCULATION OF DEVIATION FROM NORMAL BASAL METABOLISM

The basal metabolism varies according to age, size, and sex according to rules that are well enough established to permit prediction of the normal

basal metabolism with error not exceeding ± 10 per cent. In persons of the same age and sex it appears to be closely related to the surface area of the subject. Figure 24 is a d'Ocagne nomogram (19, 20) which permits the

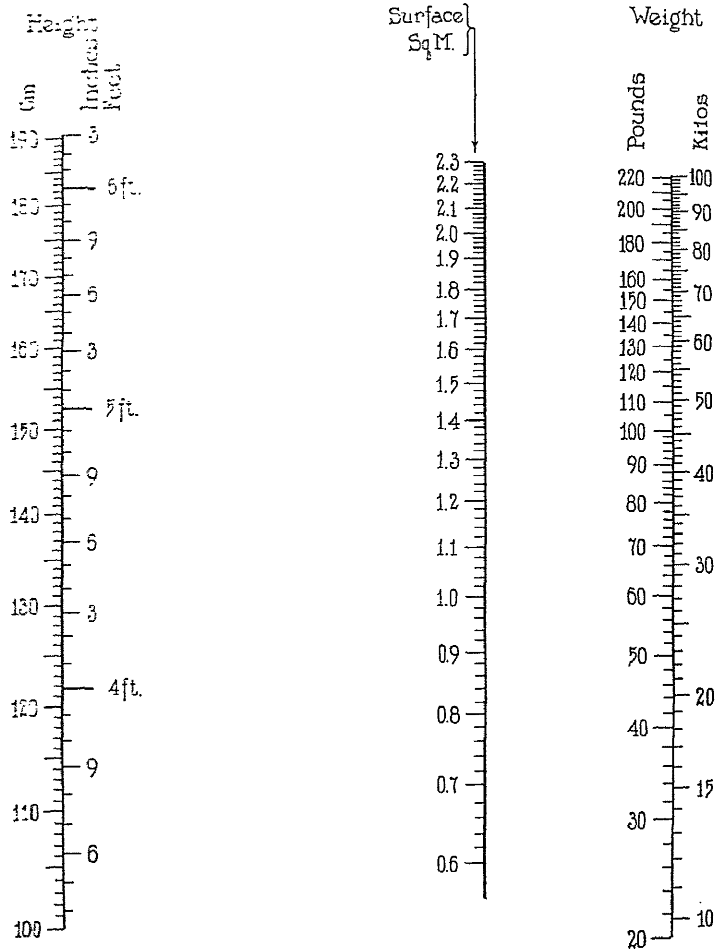


FIG. 24. Nomogram permitting direct estimation of surface area from height and weight by Du Bois' formula $A = H^{0.725} \times W^{0.425} \times 71.84$. When A = surface area in square centimeters, H = height in centimeters and W = weight in kilos. (sq. cm. = sq. m. $\times$ 10,000.) The surface area is found at the point of intersection of the middle scale with a straight line drawn from the observed height on the left hand scale to the observed weight on the right hand scale.

direct estimation of surface area from height and weight without the need of arithmetical calculation.

Table 20 shows the average normal basal metabolism per sq. meter at different ages in both sexes.[5]

TABLE 20

AVERAGE BASAL METABOLISM PER SQUARE METER ABOVE THE AGE OF FIVE. BOOTHBY AND SANDIFORD (21). MODIFICATION OF THE DU BOIS STANDARDS

AGE*	MALES	FEMALES	AGE	MALES	FEMALES
5	(53.0)	(51.5)	20	41.0	36.5
6	53.0	50.5	21	40.5	36.5
7	52.0	49.5	22–24	40.0	36.5
8	51.0	48.0	25–29	39.5	36.0
9	50.0	46.5			
10	49.0	45.5	30–34	39.0	35.5
11	48.5	44.5	35–39	38.5	35.0
12	47.5	43.0	40–44	38.0	35.0
13	47.0	42.0	45–49	37.5	34.5
14	46.0	41.0			
15	45.0	39.5	50–54	37.0	34.0
16	44.0	38.5	55–59	36.0	34.0
17	43.5	37.5	60–64	35.5	33.5
18	42.5	37.0	65–70+	35.0	33.0
19	42.0	36.5			

* In using table the age should be determined to the nearest year. That is, 4 years 6 months to 5 years 5 months inclusive, is taken as 5 years.

$$\frac{100 \times \text{(observed calories per sq. meter per hour)}}{\text{Average normal calories per sq. meter per hour}} - 100 = \text{per cent above or}$$
below average normal basal metabolism.

[5] Until recently the standard in use for normal basal metabolic rate per square meter body surface has been the table published in 1917 by Aub and Du Bois (1). It was not based on a large number of cases, particularly in some of the age groups, and the values for females were not taken from direct determinations, but were calculated as 7 per cent below males. The data did not apply to subjects below the age of fourteen. Boothby and Sandiford's values, given in table 20, were published in 1929, from observations on 1800 males and 5000 females, and cover subjects from five years upwards. The Boothby and Sandiford values are 1 to 4 per cent lower than those of Aub and Du Bois for adults, and 3 to 7 per cent lower for children. Aub and Du Bois' values were presented by them not as final, but to fill a need at the time, until, as they stated, "changes are made by the addition of new data." We have accordingly given in table 20 only the new standards of Boothby and Sandiford.

TABLE 21

Harris-Benedict Basal Metabolic Rate Standards Based on Body Weight, Sex, Stature and Age (30). For Men, Women, and Boys Above Ten

The average normal B. M. R. in calories per hour is obtained by adding the calories per hour component in Section A based on weight to the component in Section B, C, or D, based on sex, age, and stature.

TABLE 21A

Component Based on Body Weight

Weight	Calories per hour		Weight	Calories per hour	
	Males	Females		Males	Females
kgm.			kgm.		
10	8.5		72	44.0	56.0
12	9.7		74	45.2	56.8
14	10.8		76	46.3	57.6
16	12.0		78	47.5	58.4
18	13.1		80	48.6	59.2
20	14.3		82	49.7	60.0
22	15.4		84	50.9	60.8
24	16.6		86	52.0	61.6
26	17.7	37.6	88	53.2	62.4
28	18.8	38.4	90	53.3	63.2
30	19.9	39.2	92	55.5	64.0
32	21.1	40.0	94	56.6	64.8
34	22.2	40.8	96	57.8	65.6
36	23.4	41.6	98	58.9	66.4
38	24.5	42.4	100	60.1	67.2
40	25.7	43.2	102	61.2	68.0
42	26.8	44.0	104	62.4	68.8
44	28.0	44.8	106	63.5	69.6
46	29.1	45.6	108	64.7	70.4
48	30.3	46.4	110	65.8	71.2
50	31.4	47.2	112	67.0	72.0
52	32.6	48.0	114	68.1	72.8
54	33.7	48.8	116	69.3	73.6
56	34.9	49.6	118	70.4	74.4
58	36.0	50.4	120	71.6	75.2
60	37.2	51.2	122	72.7	76.0
62	38.3	52.0	124	73.9	76.8
64	39.5	52.8	126	75.0	77.6
66	40.6	53.6	128	76.1	78.4
68	41.8	54.4	130	77.2	79.2
70	42.9	55.2			

Benedict and his associates prefer to use prediction standards based on age, weight or height, and sex. Tables of such prediction standards are given with directions for their use in table 21, 22, and 23. These tables cove infants as well as children and adults.

The use of the 2 systems of calculation is illustrated by the following example. A man of 25 years, 60 kilos weight, and 170 cm. height shows a basal metabolic rate of 70 calories per hour.

Surface area standard. By figure 24 the surface area is found to be 1.68 square meters. The calories per hour per square meter are therefore

TABLE 21B

COMPONENTS BASED ON AGE AND STATURE—MEN

HEIGHT	CALORIES PER HOUR FOR AGE INDICATED										
	20	25	30	35	40	45	50	55	60	65	70
cm.											
150	25.6	24.2	22.8	21.4	20.0	18.6	17.2	15.8	14.4	13.0	11.6
155	26.6	25.2	23.8	22.4	21.0	19.6	18.2	16.8	15.4	14.0	12.6
160	27.7	26.3	24.9	23.5	22.1	20.7	19.3	17.9	16.5	15.1	13.7
165	28.7	27.3	25.9	24.5	23.1	21.7	20.3	18.9	17.5	16.1	14.1
170	29.8	28.4	27.0	25.6	24.2	22.8	21.4	20.0	18.6	17.2	15.8
175	30.8	29.4	28.0	26.6	25.2	23.8	22.4	21.0	19.6	18.2	16.8
180	31.9	30.4	29.1	27.6	26.2	24.8	23.4	22.0	20.6	19.2	17.8
185	32.9	31.5	30.1	28.7	27.3	25.9	24.5	23.1	21.7	20.3	18.9
190	34.0	32.5	31.2	29.7	28.3	26.9	25.5	24.1	22.7	21.3	19.9
195	35.0	33.6	32.2	30.8	29.4	28.0	26.6	25.2	23.8	22.4	21.0
200	36.1	34.6	33.2	31.8	30.4	29.0	27.6	26.2	24.8	23.4	22.0

$70 \div 1.68 = 41.7$. By table 20 the average normal basal metabolic rate for a male of 25 years is found to be 39.5 calories per hour per square meter. Hence the deviation from normal is calculated as $\frac{100 \times 41.7}{39.5} - 100 = 105.5 - 100 = +5.5$ per cent above average normal.

Harris-Benedict standards. The average basal metabolism normal for the subject's weight etc. is calculated as $37.2 + 28.4 = 65.6$ calories per hour. The component 37.2 is taken from Table 21A, and the component 28.4 from 21B. The deviation is calculated as $\frac{100 \times 70}{65.6} \quad 100 = 106.6 - 100 = +6.6$ per cent above average normal.

Deviations from normal calculated by the two systems differ slightly,

but as a rule not significantly. The use of one or the other system is largely a matter of convenience.

TABLE 21c

Components Based on Age and Stature—Women

Height	Calories per hour for age indicated										
	20	25	30	35	40	45	50	55	60	65	70
cm.											
150	7.7	6.7	5.7	4.7	3.8	2.8	1.8	0.9	0.0	−1.0	−2.0
155	8.1	7.1	5.1	5.1	4.2	3.2	2.2	1.2	0.2	−0.7	−1.7
160	8.5	7.5	6.5	5.5	4.5	3.6	2.6	1.6	0.6	−0.3	−1.3
165	8.8	7.8	6.9	5.9	4.9	4.0	3.0	2.0	1.0	0.0	−0.9
170	9.2	8.2	7.3	6.3	5.3	4.3	3.4	2.4	1.4	0.5	−0.5
175	9.6	8.6	7.6	6.7	5.7	4.7	3.7	2.8	1.8	0.8	−0.2
180	10.0	9.0	8.0	7.0	6.1	5.1	4.1	3.2	2.2	1.2	0.2
185	10.4	9.4	8.4	7.5	6.5	5.5	4.5	3.5	2.6	1.6	0.6
190	10.8	9.8	8.8	7.8	6.8	5.9	4.9	3.9	3.0	2.0	1.0
195	11.2	10.2	9.2	8.2	7.2	6.2	5.3	4.3	3.3	2.4	1.4
200	11.5	10.5	9.6	8.6	7.6	6.7	5.7	4.7	3.7	2.7	1.8

TABLE 21d

Components Based on Age and Stature—Boys

Height	Calories per hour for age indicated			Height	Calories per hour for age indicated		
	10	15	20		10	15	20
cm.				cm.			
100	18.0	16.6	15.2	155	29.5	28.1	26.6
105	19.0	17.7	16.3	160	30.5	29.1	27.7
110	20.0	18.7	17.3	165	31.6	30.1	28.7
115	21.0	19.7	18.3	170	32.6	31.2	29.8
120	22.1	20.8	19.4	175	33.6	32.2	30.8
125	23.2	21.8	20.4	180	34.7	33.3	31.9
130	24.2	22.9	21.5	185	35.7	34.3	32.9
135	25.3	23.9	22.5	190	36.8	35.4	34.0
140	26.3	25.0	23.6	195	37.8	36.4	35.0
145	27.4	26.02	24.6	200	38.9	37.4	36.1
150	28.4	27.0	25.6				

General theoretical consideration of the calculation of metabolism determinations can be found in the chapter on total metabolism of volume I.

TABLE 22

BENEDICT-TALBOT AVERAGE NORMAL BASAL METABOLIC RATE FOR INFANTS AND CHILDREN. CALORIES PER HOUR BASED ON BODY WEIGHT ALONE

WEIGHT	BOYS	GIRLS	WEIGHT	BOYS	GIRLS
kgm.	*cal.*	*cal.*	*kgm.*	*cal.*	*cal.*
3	6.3	6.3	.31	47.5	44.6
4	8.8	9.2	32	48.3	45.4
5	11.3	11.9	33	49.2	46.4*
6	13.8	14.6	34	50.0	47.3*
7	16.3	16.9	35	50.8	48.3*
8	18.5	19.2	36	51.7	49.2*
9	20.6	20.8	37	52.3	50.1*
10	22.7	22.5	38	53.1	50.7
11	24.6	24.2	39	For boys apply Harris-Benedict Standards A + D	50.8
12	26.0	25.4	30		51.0
13	27.5	26.7	41		51.2
14	29.0	27.7	42		51.3
15	30.2	28.8	43		51.4
16	31.5	29.6	44		51.6
17	32.5	30.6	45		51.7
18	33.5	31.7	46		51.8
19	34.6	32.5	47		52.0
20	35.8	33.5	48		52.1
21	36.9	34.6	49		52.3
22	37.9	35.6	50		52.4
23	39.2	36.7	51		52.5
24	40.2	37.5	52		52.7
25	41.3	38.8	53		52.8
26	42.5	39.6	54		52.9
27	43.5	40.6	55		53.1
28	44.6	41.7	56		53.2
29	45.4	42.5	57		53.3
30	46.5	43.5	58		53.5

* Figures obtained by interpolation. Not in original.

TABLE 23

BASAL HEAT PRODUCTION PER HOUR OF GIRLS (BENEDICT) FROM THE FIRST WEEK AFTER BIRTH TO TWELVE YEARS OF AGE. PREDICTED FROM HEIGHT ALONE (9)

HEIGHT	PREDICTED HEAT	HEIGHT	PREDICTED HEAT	HEIGHT	PREDICTED HEAT
cm.	*cals.*	*cm.*	*cals.*	*cm.*	*cals.*
48	5.08	79	24.29	110	32.04
49	5.67	80	24.42	111	32.42
50	6.25	81	24.63	112	32.83
51	6.88	82	24.79	113	33.21
52	7.42	83	24.92	114	33.63
53	8.08	84	25.08	115	34.04
54	8.67	85	25.21	116	34.50
55	9.25	86	25.29	117	34.88
56	9.83	87	25.42	118	35.29
57	10.42	88	25.50	119	35.71
58	11.17	89	25.63	120	36.08
59	11.79	90	25.71	121	36.46
60	12.50	91	25.83	122	36.88
61	13.25	92	25.96	123	37.25
62	13.83	93	26.08	124	37.67
63	14.58	94	26.25	125	38.13
64	15.29	95	26.54	126	38.54
65	16.00	96	26.83	127	38.96
66	16.71	97	27.13	128	39.38
67	17.42	98	27.46	129	39.83
68	18.13	99	27.79	130	40.21
69	18.83	100	28.13	131	40.63
70	19.50	101	28.54	132	41.04
71	20.13	102	28.88	133	41.46
72	20.83	103	29.17	134	41.88
73	21.50	104	29.63	135	42.33
74	22.08	105	30.00	136	42.75
75	22.63	106	30.42	137	43.21
76	23.21	107	30.83	138	43.63
77	23.63	108	31.21	139	44.05
78	23.96	109	31.63	140	44.47

BIBLIOGRAPHY

1. AUB, J. C., AND DU BOIS, E. F.: Clinical calorimetry. XIX. The basal metabolism of old men. *Arch. Int. Med.*, 1917, 19, 823.
2. BAILEY, C. V.: Apparatus used in the estimation of basal metabolism. *J. Lab. Clin. Med.*, 1921, 6, 657.
3. BAILEY, C. V.: Notes on apparatus used in determining the respiratory exchange in man. I. An adaptation of the French gas mask for use in respiratory work. *J. Biol. Chem.*, 1921, 47, 277.

4. BAILEY, C. V.: Notes on apparatus used in determining the respiratory exchange in man. II. A sampling bottle for gas analysis. *J. Biol. Chem.*, 1921, **47**, 281.

5. BAUER, V., AND BLUNT, K.: Effect of a small breakfast on the energy metabolism of children. *J. Biol. Chem.*, 1924, **59**, 77.

6. BENEDICT, C. G., AND BENEDICT, F. G.: A permissible breakfast prior to basal metabolism measurements. *Boston Med. Surg. J.*, 1923, **188**, 849.

7. BENEDICT, F. G.: An apparatus for studying the respiratory exchange. *Am. J. Physiol.*, 1909, **24**, 345.

8. BENEDICT, F. G.: A study of prolonged fasting. Carnegie Institute of Washington, 1915, Publication No. 203.

9. BENEDICT, F. G.: Physical factors in predicting the basal metabolism of girls. *Proc. Am. Philosophical Soc.*, 1924, **63**, 25.

10. BENEDICT, F. G.: The control of gaseous metabolism apparatus. *Boston Med. Surg. J.*, 1925, **193**, 583.

11. BENEDICT, F. G.: Some techniques for measurement of the gaseous metabolism of human beings. *Boston Med. Surg. J.*, 1925, **193**, 807.

12. BENEDICT, F. G., AND CARPENTER, T. M.: Metabolism and energy transformation of healthy men during rest. Carnegie Institute of Washington, 1910, Publication No. 126.

13. BENEDICT, F. G., AND CARPENTER, T. M.: Food ingestion and energy transformation with special reference to the stimulating effects of nutrients. Carnegie Institute of Washington, 1918, Publication No. 261.

14. BENEDICT, F. G., AND COLLINS, W. E.: A clinical apparatus for measuring basal metabolism. *Boston Med. Surg. J.*, 1920, **183**, 449.

15. BENEDICT, F. G., AND CROFT, E. E.: Is prolonged bed rest a prerequisite for the measurement of basal metabolism? *Am. J. Physiol.*, 1925, **74**, 369.

16. BENEDICT, F. G., AND TALBOT, F. B.: Metabolism and growth from birth to puberty. Carnegie Institute of Washington, 1921, Publication No. 302.

17. BOOTHBY, W. M., AND SANDIFORD, I.: Laboratory manual of the technic of basal metabolic rate determinations. W. B. Saunders, Co., Philadelphia and London, 1920.

18. BOOTHBY, W. M., AND SANDIFORD, I.: Basal metabolism. *Physiol. Rev.*, 1924, **4**, 69.

19. BOOTHBY, W. M., AND SANDIFORD, R. B.: Nomographic charts for metabolic rate determinations. *Am. J. Physiol.*, (Proceedings), 1921, **55**, 296.

20. BOOTHBY, W. M., AND SANDIFORD, R. B.: Nomographic charts for the calculation of the metabolic rate by the gasometric method. *Boston Med. Surg. J.*, 1921, **185**, 337.

21. BOOTHBY, W. M., AND SANDIFORD, I.: Normal values of basal or standard metabolism. A modification of the Dubois standards. *Proc. 13th Internat. Physiol. Congress*, 1929, p. 32.

22. CARPENTER, T. M.: A comparison of methods for determining the respiratory exchange of man. Carnegie Institute of Washington, 1915, Publication No. 216.

23. CARPENTER, T. M.: Tables, factors and formulas for computing respiratory exchange and biological transformations of energy. Carnegie Institute of Washington, 1921, Publication No. 303.

24. CARPENTER, T. M., AND FOX, E. L.: Absence of stratification and rapidity of mixing of carbon dioxide in air samples. *J. Biol. Chem.*, 1927, **73**, 379.

25. DOUGLAS, C. G.: A method for determining the total respiratory exchange in man. *J. Physiol.*, (Soc. Proc.) 1911. **42**, xvii.

26. DU BOIS, D., AND DU BOIS, E. F.: Clinical calorimetry. V. The measurement of the surface area of man. *Arch. Int. Med.*, 1915, **15**, 868.

27. DU BOIS, E. F.: Basal metabolism in health and disease. Lea and Febiger, Philadelphia, 1927. 2nd edition.

28. HAGEDORN, H. C.: An apparatus for the graphic recording of oxygen consumption and carbon dioxide output, especially adapted for clinical work. *Biochem. J.*, 1924, **18**, 1301.

29. HANNON, R. R., AND LYMAN, R. S.: A graphic method for recording oxygen consumption. *Johns Hopkins Hosp.* Bull., *1923*, **34**, *241*.

29-a. HANRIOT, M. M., AND RICHET, C.: Des éschanges respiratoires chez l'homme. *Ann. chem. phys.*, 1891. (Series VI). **22**, 495.

30. HARRIS, J. A., AND BENEDICT, F. G.: A biometric study of basal metabolism in man. Carnegie Institute of Washington, 1919, Publication No. 279.

31. KING, J. T., JR.: Determination of the basal metabolism from the carbon-dioxide elimination. *Johns Hopkins Hosp. Bull.*, 1921, **32**, 277.

32. KING, J. T., JR.: Basal Metabolism, Baltimore, 1924.

33. KROGH, A.: Ein Respirationsapparat zur klinischen Bestimmung des Energieumsatzes des Menschen. *Wien. klin. Woch.*, 1922, **35**, 290.

34. KROGH, A.: Determination of standard (basal) metabolism of patients by a recording apparatus. *Boston Med. Surg. J.*, 1923, **189**, 313.

35. LUSK, G.: Animal calorimetry. XXIV. Analysis of the oxidation of mixtures of carbohydrate and fat. A correction. *J. Biol. Chem.*, 1924, **59**, 41.

36. MCCANN, W. S.: Calorimetry in medicine, *Medicine*, 1924, **3**, 1.

37. MCCLENDON, J. F., HILDING, C. A.; STEGGERDA, F. R.; CONKLIN, C., AND WHITAKER, M.: A simple respiration apparatus for determination of oxygen and carbon dioxide in indirect calorimetry. *J. Biol. Chem.*, 1928, **77**, 413.

38. ROTH, PAUL: Modifications of apparatus and improved technic adaptable to the Benedict type of respiration apparatus. Paper I. Valves versus the electric impeller. *Boston Med. Surg. J.*, 1922, **186**, 457.
Paper II. Technic, *Ibid.*, 463.
Paper III. Graphic method for the estimation of the metabolic rate. *Ibid.*, 491.
Paper IV. Moisture absorbing efficiency of carbon dioxide absorbents. *Ibid.*, 498.

39. SODERSTROM, G. F., BARR, D. P., AND DU BOIS, E. F.: Clinical calorimetry, XXVI. The effect of a small breakfast on heat production. *Arch. Int. Med.*, 1918, **21**, 613.

40. TISSOT, J.: Nouvelle méthode de mesure et d'inscription du débit et des mouvements respiratoires de l'homme et des animaux. *J. Physiol. path. gen.*, 1904, **6**, 688.

41. WILSON, R. E.: Soda lime as an absorbent for industrial purposes. *J. Ind. Eng. chem.* 1920, **12**, 1000.
Note on moisture absorbing efficiency of carbon dioxide absorbents for metabolism apparatus. *Boston Med. Surg. J.*, 1922, **187**, 133.

CHAPTER VI

Lung Volume

The volume of air which is held in the lungs when they are filled to the limit, the "total capacity," is composed of two fractions: 1, that which can expired by a maximal expiration, the "vital capacity;" 2, that which remains in the lungs after a maximal expiration, the "*residual air.*" The vital and total capacities have been divided into various other subdivisions (5) which will not be discussed here. The vital capacity can be determined by simply observing the excursions of a spirometer. For the residual air in the lungs, however, special methods must be used. The clinical interest in the residual air is chiefly based on the fact that this unexpirable air volume increases in various conditions in which the elasticity of the lungs appears to be impaired: such increase has been noted in emphysema (8, 9) and in a considerable proportion of cases of cardiac failure (1) and tuberculosis (2). Pulmonary edema on the other hand appears to diminish not only the vital capacity (6) but also the residual air (1); consequently when cardiac failure is accompanied by pulmonary edema the residual air volume may be even less than normal.

The residual air varies from 1 to 1.5 liters in normal women, and from 1.5 to 2.5 in normal men (5), following more or less closely the height and the chest size measured in three dimensions (5).

The "lung volumes" determined by the methods described below comprise in each case the volume of air held in the lungs *and* the respiratory passages at the time the determination is begun. Also whatever dead space lies between the cock of the mouth-piece and the mouth-piece itself is included, unless corrected for.

I. DILUTION METHOD FOR SUBJECTS CAPABLE OF FORCED BREATHING. LUNDGSGAARD AND VAN SLYKE (5)

Principle

If the subject is able to cooperate by breathing deeply the procedure is simple. He merely mixes the air in his lungs with a measured volume; e.g., 2 liters of oxygen, in a bag by five or six deep breaths, which suffice to obtain a homogeneous mixture (4, 5). The N_2 content of the mixed gases is then determined by gas analysis. If the total gas volume in the lungs + bag

system remains constant, the extent to which the N_2 concentration is diminished by dilution of the pulmonary air with 2 liters of oxygen can be used to calculate the volume of air in the lungs. Approximate constancy of total gas volume can be assumed during the fifteen or twenty seconds in which five deep breaths can ordinarily be taken, the difference between O_2 absorbed and CO_2 evolved being of negligible effect during this interval.

Procedure

Lundsgaard and Van Slyke (5) applied the principle as follows. (For a review of its former application see their paper (5).) A 4 or 5-liter rubber bag connected to a three-way respiratory stop-cock and a mouthpiece (fig. 15, 1) is filled with a measured volume, usually 2 liters, of pure oxygen. (A device like that in figure 20, V, (p. 192) can be used for measuring the oxygen.) The subject closes his lips around the mouthpiece, and his nose is closed by a clamp. He respires normally a few times with the cock turned to connect his lungs with the outside air. Then he brings his lungs to the desired position (maximum exhalation if residual air is desired) and the cock is turned to connect him with the bag. Four or five deep respirations mix the air in the lungs with that in the bag. A sample of gas is then drawn from the bag and its nitrogen content is determined.

In case one determines not the residual air, but the larger volume of air in the lungs at the end of a normal inspiration or expiration, it is preferable to use 3 liters of oxygen, rather than 2, in the bag. The results are most accurate when the volume of oxygen placed in the bag approximates the volume of air in the lungs.

Analysis

The gas usually contains less than 50 per cent of nitrogen, so that a Haldane apparatus with the type of gas burette used for analysis of of expired air (fig. 10) can not be used. A straight burette permitting readings of all percentages of gases must be substituted.

Since there is no limitation in the size of samples and 0.1 per cent accuracy suffices, analyses may be made with an ordinary 50 cc. gas burette and Hempel pipette, as done by Lundsgaard and Van Slyke (5) and Binger (1, 8).

A convenient and more accurate procedure is the manometric nitrogen determination described on page 118.

Calculation

$$\text{Lung volume} = V_{O_2} \times \frac{\text{per cent N}_2}{79.1 - \text{per cent N}_2}$$

V_{O_2} is the volume of oxygen measured into the bag at the start and "per cent N_2" represents the per cent of nitrogen found in the mixed gases at the end of the experiment.

The oxygen used must be analyzed for nitrogen by the same reagents used in analyzing the respired mixture. If it contains a per cent of impurity thus determined as nitrogen, the calculation formula is modified to:

$$\text{Lung volume} = V_{O_2} \times \frac{\text{per cent N}_2 - a}{79.1 - \text{per cent N}_2}$$

The *determination of small amounts of N_2 in oxygen* can be made most accurately with the manometric apparatus as described on page 119, the pressure of the sample being measured at 50 cc. volume and the pressure of unabsorbed N_2 at 2 cc. volume.

The formula used for calculation is derived as follows:

Let:

V_L = Lung volume.

V_{N_2} = Volume of N_2 in lungs at beginning of experiment.

V_{O_2} = Volume of O_2 measured into bag.

i = per cent of N_2 in air of lungs at beginning of experiment. i was found by Lundsgaard and Van Slyke to vary from 78.4 to 79.6, and to average 79.1. Ordinarily i is assumed to be 79.1.*

b = per cent of N_2 in mixed air of bag at end of experiment.

$$V_{N_2} = \frac{i}{100} \times V_L = \frac{b}{100} \times (V_L + V_{O_2})$$

By equating the second and third members of this expression and solving for V_L we obtain:

$$V_L = V_{O_2} \times$$

If the oxygen contains a per cent of N_2, or of gas behaving like N_2 towards the reagents used in the analysis, the first equation becomes:

$$V_{N_2} = \frac{i}{100} \times V_L + \frac{a}{100} \times V_{O_2} = \frac{b}{100} \times (V_L + V_{O_2})$$

* Christy (personal communication) finds that the accuracy of the determination is increased if i is determined in each case by preliminary analysis of the alveolar air of the subject.

By equating the second and third members and solving for V_L we obtain:

$$V_L = V_{O_2} \times \frac{b - a}{i - b}$$

Correction for dead space in mouth piece. From the "lung volume" calculated by the above formulae one must subtract the relatively small volume of the dead space, perhaps 0.05 liter, in the tube between the valve and the mouth of the subject. It can be estimated with sufficient accuracy as $\frac{0.8\ D^2H}{1000}$ liters, where D is the diameter and H the length in in centimeters of tubing between the subject's mouth and the cock of the apparatus (fig. 15). Or this dead space may be measured by determining the volume of water required to fill it.

II. DILUTION METHOD WITHOUT FORCED BREATHING. SENDROY, HILLER, AND VAN SLYKE (7)

Principle

If the subject is unable to cooperate by making repeated deep inhalations and exhalations, complete mixture of the oxygen in the bag with the air in the lungs may require, not five breaths and fifteen seconds of time, but continued respiration for two or more minutes. To make continuous rebreathing possible without acute distress from accumulated carbon dioxide it is necessary to remove the CO_2 by absorption with a scrubber. Such removal, however, continually diminishes the volume of gas in the system. In consequence the total volume to which the pulmonary nitrogen is diluted can not be estimated from the initial volume of oxygen present in the apparatus. The apparatus must be arranged so that it shows the final volume of gas in the extrapulmonary part of the system at the end of the experiment.

Apparatus

Whatever apparatus is used, its valves and connections must meet the tests for leaks and absence of resistance described in the preceding chapter for respiratory apparatus in general.

Krogh and Roth-Benedict spirometers. Both these instruments, shown in figure 22 and 21 of the preceding chapter, serve for determination of lung volume as well as oxygen consumption. When either apparatus is used it is necessary to determine the volume, V_D of the dead space filled with gas when the spirometer bell is depressed to the zero point. This space represents the volume of gas still remaining in the soda lime scrubber and other parts of either spirometer when the bell is at zero. The value of V_D for the

apparatus used is determined, as detailed below, by means of the same dilution principle applied in procedure I for determination of the lung volume itself.

At the end of an experiment the total gas content of the apparatus is calculated as V_D plus the volume V_M registered on the scale of the spirometer. In the calculation formula therefore the volume, $V_D + V_M$, replaces V_{O_2} used in the formula for calculating results of procedure A.

Sendroy apparatus (7). In figure 23 is shown an apparatus which can be set up at an expense much less than the cost of a spirometer, and gives results equally accurate. The Sendroy apparatus has another advantage with respect to economy in that its dead space is much smaller than that of either

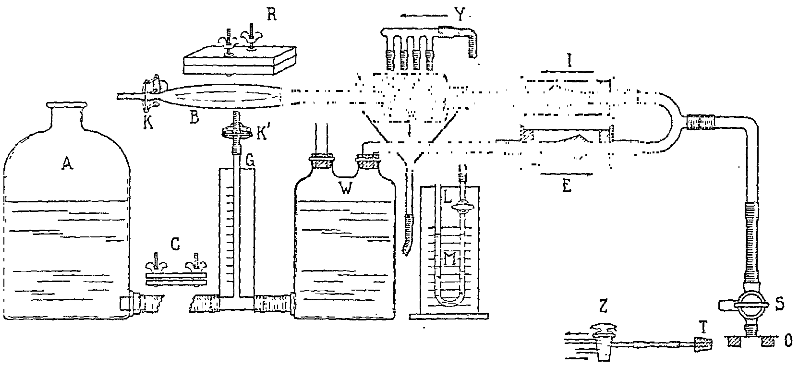


FIG. 25. Apparatus of Sendroy (7) for determination of lung volume by diluting pulmonary nitrogen with measured volumes of oxygen without forced breathing.

spirometer, so that it requires only a fraction as much oxygen to wash out other gases before each determination.

Z is a two-way stop-cock, one end of which is connected to the vacuum, the other to a high-pressure tank of pure oxygen. The rubber stopper T fits the rubber mouth piece O. The aluminum three-way stop-cock S of 20 mm. bore is made to communicate with the outside air, or through O with the apparatus. I and E are rubber spirometer valves, (inlet and outlet) enclosed in glass jackets. X is a 500 to 750 cc. bottle with bottom removed, containing a supply of soda lime. Copper gauze of not too fine mesh or cotton loosely packed, may be used to pack the particles within the bottle. Running water enters the cooling system at Y, is distributed along the sides of the bottle, and runs off into a funnel. B is a rubber breathing bag, of 5 or 6 liters capacity. R is a press, something like that used for holding tennis racquets. It consists of two pieces of wood large enough in area to cover all but the ends of the bag B. B in also in communication with the Woulfe bottle W of 8 liters capacity, containing water. G is a level gage with a millimeter scale. The bottle W is calibrated on the scale G by pouring in measured volumes of

water, 300 to 400 cc. at a time, so that definite volumes of gas in W correspond to definite scale readings of the meniscus in G. The points are plotted on a curve, from which scale readings can be converted into liters of gas present in W (1 mm. on the scale of G corresponds to about 30 cc. volume in W). Reading G to 0.5 mm. in such an apparatus gives gas volumes in W to $\pm$ 15 cc. The flow of water through a suitable length of rubber tubing (32 mm. wide) between A and W is controlled by the clamp C, placed as near as possible to the volume gage G. M is a water-filled manometer with a millimeter scale, connected at will to the rest of the system through the stop-cock L of at least 2 mm. bore. All of the connecting tubes through which respired air passes are of heavy-walled glass or rubber tubing, of an internal diameter (23 mm.) sufficiently large to avoid resistance to respiration. In order to make the rubber tube connections at the bottom outlets of A and W, it may be necessary to seal a piece of glass tubing into the outlet. This may easily be done by the use of de Khotinsky cement. No temperature control is necessary, since the change in temperature of the gas contained within the system is so small during the period of a determination that it may be neglected. The zero point at the top of scale G is 3 or 4 cm. below the bottoms of the stoppers in W.

Determination of V_D, the dead space of the apparatus

The apparatus is first filled with atmospheric air. If it has previously had any other kind of gas in it, it is filled with air and emptied 12 to 15 times to replace other gases entirely with air of atmospheric composition. Then the air content is reduced to that of the dead space, and is mixed with a known volume of oxygen, as follows.

Krogh or Roth spirometer. The bell is pressed down until the indicator points to the zero mark on the scale so that no air except that in the dead space remains in the spirometer. Then the mouth piece of the spirometer is connected with a gas container holding a known volume of oxygen. An aspirator bottle like A in figure 25 serves as such a container. It is provided at the top with a stopper and a cock by which gas can be admitted and let out, and is connected at the bottom with a similar bottle which is filled with water. A mark is made on the stoppered bottle showing the level at which water stands in it when 5 liters of gas are present. The bottle thus calibrated is first filled to the stopper with water, and then pure oxygen is run in until the water has fallen to the 5-liter mark, the level of the water in the connected flask being kept even with that in the calibrated one. The calibrated bottle is then connected with the spirometer by a short narrow tube bearing a perforated stopper which fits into the hole in the mouth piece of the spirometer. By raising and lowering the other bottle the oxygen is alternately forced into the spirometer and withdrawn from it 15 times, so that a uniform mixture of the oxygen with the air in the spirometer is obtained. A sample of the mixed gas is then analyzed for nitrogen, as in Method I.

The calculation of the dead space is similar to that of lung volume, Method I.

$$V_D = V_{O_2} \times \frac{\text{per cent } N_2 - a}{79.04 - \text{per cent } N_2}$$

In this case V_{O_2} represents the volume of oxygen measured into the bottle and then mixed with the air in the spriometer. V_{O_2} is 5 liters when the technique is carried through as above directed. "Per cent N_2" represents the per cent of N_2 found in the gas mixture by analysis. The per cent of N_2 present as impurity in the oxygen used is represented by a.

Sendroy Apparatus. After the apparatus (fig. 25) is washed out with air, the bag ***B*** is pressed in clamp ***R*** and the water in ***W*** is raised until the water is up to the zero mark on scale ***G***. A bottle containing a measured volume of pure oxygen is attached to the mouth piece, clamp ***R*** is then removed from the bag, and the rest of the determination of V_D is carried out in the same manner as with the spirometers. In this case, however, the volume of oxygen used is 1 instead of 5 liters, and an aspirator bottle of 2 liters capacity is calibrated to hold the oxygen.

Washing gases other than oxygen out of apparatus before the determination

Roth and Krogh spirometers. Either of these instruments is filled as completely as possible with oxygen and emptied 12 to 15 times to remove all nitrogen. The washing of these spirometers requires about 90 liters of oxygen.

Sendroy apparatus. ***W*** is nearly filled with water and clamp ***C*** is closed. Then the stopper ***T*** is inserted into the hole in the mouth piece. One outlet of the three-way cock ***Z*** connects with a suction pump and the other with an oxygen tank. Suction is applied through ***Z*** until the bag ***B*** is nearly deflated. Strong negative pressure is not applied because it might start leaks in the apparatus. By turning ***Z*** enough oxygen to fill the gas bag is alternately admitted and withdrawn 10 times. The washing can thus be completed in two or three minutes, and requires about 40 liters of oxygen.

Addition of oxygen for the determination

With the dead space already filled with pure oxygen, sufficiently more is run into the apparatus to suffice for the requirements of the subject for

the duration of the experiment. For a resting subject 3 liters for five minutes and 6 liters for 10 provide more than enough.

With the *Krogh* or *Roth* apparatus one merely admits 3 or 6 liters of oxygen, measured by the rise of the pointer on the scale, for a five or a ten-minute period.

With the *Sendroy* apparatus the oxygen is admitted as follows. The bag *B* (fig. 25) is flattened by clamping it with *R*. *T* is then inserted into the mouth piece, *S*, and enough oxygen is wasted through cock *S* into outer air to wash the air out of this cock. Then oxygen is admitted into the apparatus until the water level in *W* has fallen to a point indicating that the desired amount of gas has been admitted. This level can be previously determined and indicated by a mark on bottle *W*. (The measurement need be only an approximate one.) The clamp is then removed from the bag and the latter is filled with gas from *W*. Clamp *C* is then closed, and bottle *A* is left elevated above *W*.

The respiration period

The determination is carried out in the same way with any of the three types of apparatus.

The subject, with nostrils clamped by a nose piece, is connected to the apparatus by means of the mouth piece, and the cock, *S* is so turned that room air is breathed for several normal respirations. The subject then brings his lungs to the desired position (for residual air he expires as completely as possible), and the cock is turned to connect him with the spirometer or bag. The subject then respires normally for the desired period. Five minutes usually suffice for complete mixture even with ill patients. Longer periods may perhaps be needed when the respirations are very shallow. At the end of the period the subject brings his lungs to the same position as at the beginning.

In the Sendroy apparatus it is necessary to admit additional gas into the bag from *W* at intervals during the period. This is done by opening clamp *C* and admitting water from *A* into *W*. So much must not be admitted at any time that the bag is sufficiently filled to offer resistance to expiration. In particular, when residual air is to be determined, enough space must be left in the bag to receive the final maximal expiration.

Final gas measurements and sampling

In the Krogh or Roth spirometer the gas volume is read on the scale. The total gas volume in the extrapulmonary part of the system is then

calculated by adding the volume, V_M, indicated by the scale, to the dead space, V_D, previously determined. A sample of gas from the spriometer is then drawn for gas analysis, in the manner discussed under "Sampling" in chapter III.

With the Sendroy apparatus the following procedure is followed. The bag is compressed in the clamp **R** and the gas is thereby completely driven from the bag into *W*, as it was when the dead space was determined. The volume V_M is then read on the scale *G*. During the reading the cock of **M** is opened and the bottle **A** is set at such a level that the water menisci in the two limbs of manometer *M* are at the same level, indicating exact atmospheric pressure in the system.

To obtain a sample the bag is then refilled with gas from *W*, and the sample is withdrawn from outlet *K* of the bag.

Analysis

The remarks on analysis made under Method I apply also here. Since the greater dilution of the pulmonary air brings the nitrogen content in the present case down to 12 or 15 per cent, accuracy in the analysis is more essential than in Method I. The manometric analysis (p. 118) has proven satisfactory (7).

Calculation

The calculation is the same as in Method I. Here the volume of extrapulmonary gas with which the nitrogen of the pulmonary air is diluted is indicated by the total gas volume, $V_M + V_D$, in the spirometer at the end of the respiration period.

$$\text{Lung volume} = (V_M + V_D) \times \frac{N_2}{79.1 - N_2}$$

V_M represents the gas volume read at the end of the period on the scale of the spirometer or of the Sendroy apparatus, V_D is the dead space of the apparatus used, and N_2 is the per cent of nitrogen found in the gas at the end of the test.

If the oxygen used is found on analysis, by the same method used for the respired mixture, to contain a significant proportion of nitrogen, the calculation is modified to:

$$\text{Lung volume} = \frac{V_D\,(N_2 - a) + V_{M_2} N_2 - V_{M_1}}{79.1 - N_2}$$

V_D is, as above, the dead space of the apparatus; N_2 is the per cent of nitrogen found in the gas at the end of the test; a is the per cent of nitrogen found as impurity in the oxygen used; V_{M_1} represents the volume of gas read on the scale at the beginning of the test; V_{M_2} is the volume of gas read on the scale at the end of the test.

Correction for the dead space in the mouth piece is made as described under "calculation" for Method I.

III. HYDROGEN METHOD WITHOUT FORCED BREATHING. VAN SLYKE AND BINGER (10)

Principle

This procedure depends upon mixing the air in the lungs with an oxygen-hydrogen mixture containing a *known volume of hydrogen.* After complete mixing the N_2:H_2 ratio is determined by analysis, and from this ratio the volumes of N_2 and of air that were in the lungs are calculated. Because neither N_2 nor H_2 participates to a significant extent in the respiratory exchange, respiration can be continued for as long as is necessary to secure complete mixing.

This method serves the same purpose as Method II, and has over the latter two advantages: 1, the dead space of the apparatus here need not be determined; 2, the subject does not have to bring his lungs at the end of the period to the position taken at the start. The results are therefore free from whatever errors may be involved in the estimation of the dead space and in variability in the final bringing of the lungs back to position. On the other hand the use of an oxygen-hydrogen mixture demands certain precautions, discussed below, which are not necessary with Method II. Both procedures are carried out with the same apparatus, so that the choice depends upon whether the facilities for obtaining satisfactory hydrogen, and for analyzing, testing, and handling it, are such that the precautions required by the hydrogen method counterbalance its advantages.

Washing gases other than oxygen out of the spirometer or Sendroy apparatus

This is done exactly as in Method II. The apparatus is left containing as much oxygen as it will hold, except for space left for hydrogen.

Measurement of hydrogen into apparatus

From a calibrated bulb or other vessel a measured volume of hydrogen, usually 2 liters, is run into the spirometer or Sendroy apparatus.

Respiratory period

This is carried out exactly as in Method II.

Analysis of Respired Gas Mixture

A sample of the mixed gases is then freed of CO_2 and O_2 by shaking in a sampling tube or Hempel pipette with alkaline hyposulfite or pyrogallol solution. The remaining hydrogen-nitrogen mixture is then analyzed by determining the H_2 content by one of the methods described in chapter III.

Calculation

All the nitrogen in the final gas mixture comes from the air which was in the lungs and in the dead space between valve and mouth piece at the beginning, since the bag or spirometer contained only oxygen and hydrogen. One therefore calculates the volume of nitrogen present, and multiplies this by 1/0.791, or 1.264, to obtain the volume of air that was in the lungs and dead space when the subject was connected with the rebreathing apparatus.

$$\text{Liters } N_2 \text{ (from lungs and mouth piece) in gas mixture} = \text{liters } H_2 \times \frac{\text{per cent } N_2}{\text{per cent } H_2}.$$

$$\text{Liters air in lungs and dead space} = 1.264 \times \text{liters } N_2.$$

If the oxygen used contains significant amounts of N_2, the "*liters* N_2" calculated as above indicated must be corrected by *subtracting the volume of* N_2 *present as impurity in the oxygen used;* e.g., if 10 liters of oxygen are contained in the total apparatus at the beginning of the test, and the N_2 content is 0.5 per cent, the correction to subtract from "liters N_2" in the above formula is $\frac{0.5}{100} \times 10 = 0.05$ liters, and the correction for "liters air in lungs" would be $0.05 \times 1.264 = 0.06$ liters in the lung volume found. The correction is not very important, considering that the "residual air" is from 1 to 2.5 liters, but it would in this case be enough to be included in accurate work. In making this correction one must estimate the total oxygen volume in the apparatus at the *beginning* of the respiration period, viz., the volume registered by the scale of the Krogh or Roth spirometer or the Sendroy apparatus plus the volume in the dead space, V_D, which is measured as described under Method II.

If the oxygen used is contaminated with significant amounts of hydrogen, or the hydrogen with oxygen, either gas can be freed of the other by passage through a tube containing heated platinized asbestos.

Correction for the dead space in the mouth-piece is made as described for Method II.

Precautions

During the experiment the spirometer and lungs of the subject are filled with an *explosive oxygen-hydrogen mixture.* No flames or burning cigarettes should be near. No combustible grease should be used as lubricant anywhere in the apparatus, because mere contact of such grease with gas of high oxygen content may cause spontaneous combustion.

The *hydrogen used must be tested for the presence of arsenic* by means of the usual "arsenic mirror" test. *Use of hydrogen which contains arsine could be fatal.* It is preferable to use commercial hydrogen made by electrolysis rather than hydrogen made by the action of acid on zinc, because most zinc contains traces of arsenic, and when treated with acid yields traces of arsine mixed with the hydrogen.

BIBLIOGRAPHY

1. Binger, C. A. L.: The lung volume in heart disease. *J. Exp. Med.*, 1923, **38**, 445.
2. Garvin, A., Lundsgaard, C., and Van Slyke, D. D.: Studies of lung volume. II. Tuberculous men. III. Tuberculous women. *J. Exp. Med.*, 1918, **27**, 87 and 129.
3. Grollman, A., and Marshall, E. K., Jr.: The time necessary for rebreathing in a lung-bag mixture in order to attain a homogeneous mixture. *Am. J. Physiol.*, 1928, **86**, 110.
4. Lundsgaard, C., and Schierbeck, K.: Studies on the mixture of air in the lungs with various gases. I. *Am. J. Physiol.*, 1923, **64**, 210.
   II. The quantitative influence of certain factors in the producing a full mixture of hydrogen in the lungs. *Am. J. Physiol.*, 1923, **64**, 231.
5. Lundsgaard, C., and Van Slyke, D. D.: Studies of lung volume. I. Relation between thorax size and lung volume in normal adults. *J. Exp. Med.*, 1918, **27**, 65.
6. Peabody, F. C., and Sturgis, C. C.: Clinical studies of the respiration. VII. The effect of general weakness and fatigue on the vital capacity of the lungs. *Arch. Int. Med.*, 1921, **28**, 501.
7. Sendroy, J., Jr.; Hiller, A., and Van Slyke, D. D.: Determination of lung volume by the nitrogen dilution method without forced breathing. *J. Exp. Med.*, March, 1932.
8. Siebeck, R.: Über den Gasaustauch zwischen der Aussen luft und den Alveolen. *D. Arch. klin. Med.*, 1911, **102**, 390.
9. Siebeck, R.: Über die Beeinflüssung der Atemmechanik durch krankhafte Zustände des Respirations-und Kreislaufapparates. *D. Arch. klin. Med.*, 1910, **100**, 204.
10. Van Slyke, D. D., and Binger, C. A. L.: The determination of lung volume without forced breathing. *J. Exp. Med.*, 1923, **37**, 457.
11. Wilson, R. E.: Soda lime as an absorbent for industrial purposes. *J. Ind. Eng. Chem.*, 1920, **12**, 1000.

CHAPTER VII

Gasometric Methods for Analysis of Blood and Other Solutions

GENERAL METHODS AND APPARATUS FOR DETERMINING BLOOD GASES

Principles of methods for blood gas determinations

Oxygen, and carbon monoxide when present, exist in the blood almost entirely as hemoglobin compounds; carbon dioxide is chiefly in the form of bicarbonate. Before these gases can be readily extracted for measurement they must be freed from their combinations. All the present methods use ferricyanide, introduced for this purpose by Barcroft and Haldane (4) to free oxygen and carbon monoxide; while to decompose the bicarbonate, tartaric and lactic acids, which do not coagulate the proteins, are employed.

The extraction of the gases has been performed in two different ways. In the Haldane (4, 15) and Barcroft (4, 5, 6) methods the blood is placed in a relatively large chamber filled with air at atmospheric pressure and connected with either a manometer or a micro gas burette. Alkaline ferricyanide is used to liberate the oxygen, while retaining the carbon dioxide, and the oxygen set free is measured by the increase in the volume or in the pressure of the supernatant air in the chamber. The carbon dioxide is then liberated with tartaric acid and similarly measured. The relatively large air volume, about 40-fold that of the gas measured, necessitates careful temperature controls. The use of the alkaline ferricyanide has a disadvantage in that the alkalinity favors a slow consumption of oxygen by some reducng agent in the blood-reagent mixture, so that lower results are likely to be obtained than when the oxygen is freed in neutral or slightly acid solution (1, 64). In oxygen determination with alkaline ferricyanide by the methods of Haldane or Barcroft, one is likely to observe that the maximum increase of gas volume or pressure, reached after some time, is not maintained, but is followed by a fall due to gradual oxygen consumption. Furthermore, the observed maximum in such a case is lower than would be obtained without this disturbing factor. It is presumably on this account that the average normal oxygen capacity of the blood of adult men was found lower by Haldane (18.5 volumes per cent) than by those who have used the vacuum extraction methods described below. The Barcroft-Haldane methods have nevertheless yielded results of brilliant significance in many physiological researches, and are still in use in many laboratories.

Henderson and Smith (18) freed the gases by stirring the blood, after it had been treated with the same reagents used by Haldane and Barcroft, in a vessel filled with air. They then analyzed the air, and from its change in composition estimated the amount of CO_2 or O_2 that had entered it from the blood.

In the vacuum extraction methods described in this chapter the blood gases are freed and extracted from solution in an evacuated chamber. They are measured in the same chamber after complete or partial release of the vacuum by admission of mercury. No volume of air is present to magnify the errors of measurement and the speed of extraction (one to three minutes) together with conditions permitting the use of neutral or acid reagents to liberate oxygen, prevent measurable consumption of this gas after it is liberated. The absence of gases other than those of blood permits carbon dioxide, oxygen and carbon monoxide to be extracted together and determined by successive absorption with reagents specific for each.

The volumetric and manometric blood gas apparatuses

The volumetric apparatus (fig. 26), first described by Van Slyke (44) and later somewhat refined by Van Slyke and Stadie (38, 64) consists essentially of a 50-cc. pipette with a cock sealed to each end, the lower end being attached to a heavy-walled rubber tube connecting with a mercury leveling bulb, the upper end serving for reception of blood samples and reagents. The stem of the pipette at the upper end is calibrated in 0.02-cc. marks for a space of 1 cc. for measurements of the gases obtained. The analysis is carried out by filling the apparatus with mercury, then measuring in the blood sample, either preceded or followed by the reagents to free the gases. The latter are extracted from solution by lowering the mercury until a vacuum is obtained in the pipette, which is then shaken for one to three minutes. The liquid is then drawn off into a small bulb sealed on at the bottom, and mercury is readmitted until the pressure is restored to atmospheric. The volume of gas is read in the graduated upper stem of the pipette. When a mixture of gases is obtained carbon dioxide is absorbed with alkali, oxygen with hyposulfite, and carbon monoxide with cuprous chloride solution.

The accuracy of the readings has been increased by adding a manometer to the apparatus (48, 59). For the readings in this apparatus the gas is reduced, not to atmospheric pressure, but to a convenient definite volume, usually 0.5 or 2.0 cc., and the amount of gas present is determined from the pressure it exerts on the manometer. Determinations can be made with this apparatus with a constancy of 2 or 3 parts per 1000. The manometric apparatus is shown on pages 270–72.

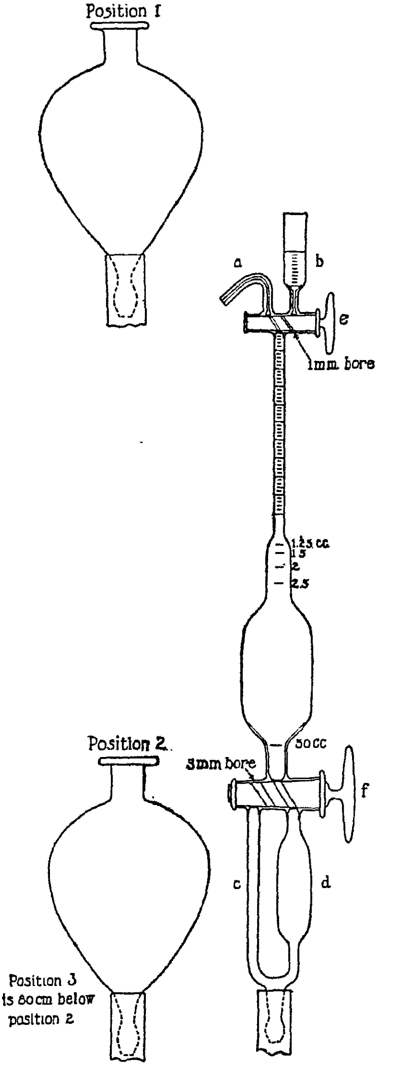


FIG. 26. Volumetric blood gas apparatus. From Van Slyke (44, 64).

For the determination of the carbon dioxide in blood or plasma as an indication of the alkali reserve, of oxygen capacity as a measure of the hemoglobin content, and of carbon monoxide content as an evidence of poisoning,

the original, simplest, "volumetric" form of the apparatus usually suffices. This is the one first described below. It permits one to determine CO_2 in 1 cc. of blood with an accuracy of 1 volume per cent, and to determine O_2 or CO in 2 cc. of blood within 0.25 volume per cent.

When a greater degree of accuracy is desired, or when all the gases are to be determined in a single 1 cc. blood sample, or when micro determinations are to be made (on 0.2 cc. of blood) the manometric apparatus is recommended. The manometric apparatus, on account of its accuracy over a great range of measurements, is also applicable to many other gasometric analyses.

TECHNIQUE AND ACCESSORIES COMMON TO BOTH VOLUMETRIC AND MANOMETRIC APPARATUS

Accessory apparatus

In order to be equipped for all the analyses that are described in this chapter with the manometric apparatus one requires the accessories listed below. Those marked with stars are required also for blood gas analyses with the volumetric apparatus.

1 pipette, 0.1 cc. calibrated "to contain." Holds 1.345 grams of dry mercury (see p. 18–21).
1 pipette, 0.2 cc. calibrated "to contain." Holds 2.69 grams of dry mercury.
1 pipette, 0.2 cc. calibrated to deliver (see p. 18–21).
1 pipette, 0.5 cc. calibrated to deliver.
*2 pipettes, 1.0 cc. with stop-cock as in figure 30.
1 pipette, 1.5 cc. with stop-cock as in figure 30 (for blood sugar determinations only).
*2 pipettes, 2.0 cc. with stop-cock as in figure 30.
2 pipettes, 3.0 cc. with stop-cock as in figure 30.
1 pipette, 5.0 cc. with stop-cock as in figure 30.
1 to 3 modified Hempel pipettes, as in figure 11.
2 or 3 calcium chloride tubes equipped as in figure 27.
2 micro 2-cc. burettes graduated into 0.02 cc. divisions and fitted with long capillary tips, for use as in figure 52, p. 344.
1 interval timer, capable of being set to ring after 1, 2, etc., minutes (that manufactured by the Victory X-Ray Company of Chicago is satisfactory).
1 30- or 40-cc. bottle for mercury. The bottle is closed with a cork stopper through which a hole of about 1 mm. diameter is pierced with a hot wire. The bottle is used to deliver mercury, a few drops at a time, into the cup of the gas apparatus to seal the cock.
1 reading glass, for reading the manometer scale to 0.1 mm.
1 stool, about 1 foot high, to stand on when high manometer readings are taken.
1 spare extraction chamber (for manometric apparatus).

1 combustion chamber for determination of H_2, O_2, and combustible gases (see figure 15, chapter III).

GENERAL REAGENTS FOR BLOOD GAS DETERMINATIONS

Approximately 1 N lactic acid, made by diluting 1 volume of concentrated acid of 1.20 specific gravity to 10 volumes with water. This lactic acid solution should be CO_2 free, because it is used in determining CO_2 in blood. The distilled water is likely to contain a little CO_2 held, by ammonia or by alkali dissolved from glass, in the form of alkali bicarbonate. To remove such CO_2 after the water and concentrated lactic acid are mixed the solution is aerated by shaking it in a large evacuated flask or by bubbling air through it. The CO_2 content is thus reduced to about 0.03 volumes per cent, in equilibrium with atmospheric CO_2.

Approximately 0.1 N lactic acid made by diluting the above 10-fold and aerating or boiling to remove carbon dioxide.

18 N (saturated) CO_2-free sodium hydroxide. NaOH is dissolved in an equal weight of water, and the solution is permitted to stand in a paraffin lined bottle until the carbonate has settled.

5 N sodium hydroxide (20 per cent). Of the above 18 N solution 27 cc. are diluted to 100 cc. to make the 5 N solution, which is kept protected from atmospheric CO_2 in the soda-lime tube shown in figure 53, p. 363.

Approximately 1 N sodium hydroxide. Of the above described 18 N NaOH 5.5 cc. are diluted to a liter.

20 per cent sodium hyposulfite. Ten grams of powdered sodium hyposulfite ($Na_2S_2O_4$) and 1 gram of sodium anthraquinone beta-sulfonate[1] are placed in a beaker. The two solids may be conveniently mixed in large amounts. Then 11-gram portions of the mixture may be measured with sufficient accuracy by volume in a marked test tube. Fifty cubic centimeters of 1.0 N potassium hydroxide solution are poured over the pulverized reagents in the beaker and immediately stirred for a few seconds with a rod. To remove some insoluble impurity that is usually present the solution is quickly filtered through cotton. It is at once transferred to the chamber of the gas analysis apparatus, where it is freed from air, and whence it is transferred to a container under oil, as described below, or to the modified Hempel pipette shown in figures 11 and 50; the pipette in this case contains mercury to protect the hyposulfite from air. The processes of dissolving, filtering and transferring to the gas apparatus should be carried out as quickly as possible in order to avoid oxidation of the solution by the air. The anthraquinone beta-sulfo-

[1] The sodium anthraquinone beta-sulfonate may be obtained from the Eastman Kodak Company.

nate is not absolutely necessary. It so catalyzes the absorption of O_2, however, that it can be completed in 1 minute with the mixture prescribed, whereas three to four minutes would be required by hyposulfite alone. The introduction of the sulfonate is due to Fieser (9). If the solution is kept under oil (figure 27) it must be used the same day it is made. If it is completely protected from air by mercury (fig. 11) it can be used for a fortnight but not longer. When the solution deteriorates, either from oxidation or from standing, the deep red color changes to a light red or yellow.

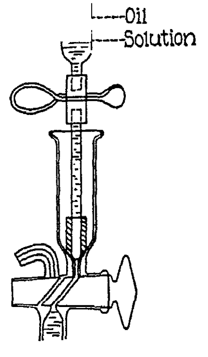


FIG. 27. Transfer of gas-free solution from extraction chamber to oil-containing tube. There should be enough oil to make a layer about 4 cm. deep.

Neutral saponin solution. Three grams of saponin are dissolved in 1 liter of water.

Neutral saponin-ferricyanide solution. Six grams of potassium ferricyanide and 3 grams of saponin (Merck) are added to 1 liter of water.

Acid saponin-ferricyanide solution. Two solutions are made: (a) 8 grams of saponin and 32 grams of potassium ferricyanide are diluted with water to 1 liter; (b) 8 cc. of concentrated lactic acid of specific gravity 1.20 are diluted to 1 liter. Before use equal volumes of the two solutions are mixed. The acid mixture gradually decomposes and some of the products settle as a blue precipitate. Consequently the combined acid mixture should be made

up in amounts sufficient only for immediate use. The mixture may be used for some days, but it is safe practice to discard the unused portion at the end of each day's work.

Winkler's cuprous chloride solution for absorbing carbon monoxide. Two hundred grams of cuprous chloride and 250 grams of ammonium chloride are dissolved in 750 cc. of water.

Pure cuprous chloride should be white. On exposure to air, moisture or light, however, it takes on a greenish tinge and becomes less soluble in ammonium chloride, because of the formation of a copper oxychloride. In order to avoid the formation of the insoluble oxychloride during the preparation of the solution, the procedure should be carried out with the exclusion of air, as far as possible. This can be done by making up the solution in a flask or bottle just large enough to contain it. A coil of copper wire which will extend throughout the depth of the solution is placed in the container. The flask or bottle is stoppered tightly with a rubber stopper as soon as the salts and water have been introduced and is then shaken to aid in their solution. The reagent is then covered with a layer of paraffin oil and kept in the same container, with the copper coil, without transfer. The oxychloride which forms will settle out fairly rapidly and the supernatant fluid can be removed as it is required.

Gas-free reagent solutions. The sodium hydroxide and hyposulfite solutions are freed of air by running about 25 cc. of solution into the 50-cc. chamber of the gas apparatus, either volumetric or manometric. The chamber is then evacuated and shaken for about three minutes. The air which has been extracted is ejected from the top of the chamber, the cock is sealed with a drop of mercury and the apparatus is evacuated and shaken again. If another bubble of air is obtained it is ejected and a third extraction performed, which in practically all cases leaves no measurable amount of dissolved air in the solution.

The air-free solutions are either transferred under oil to containers made by attaching pinch-cock tips to calcium chloride tubes, as shown in figure 27, or, much better, are stored in the modified Hempel pipettes shown in figures 11 and 50, where absolute protection from air is afforded by mercury in the upper bulb. The tip of the tube leading to either receiver is provided with a rubber ring cut from a capillary tube of soft rubber of about 1-mm. bore and 5-mm. outside diameter. The lower end of the ring is slightly beveled to fit into the bottom of the cup of the gas apparatus (fig. 27). By raising the mercury leveling bulb and opening the connecting cocks the solution is forced up into the receiver, which is already provided with oil (fig. 27) or mercury (fig. 11) to protect the solution from air. The protection af-

forded by oil is not absolute, but suffices for one morning or afternoon. The protection from air afforded by mercury in the modified Hempel pipette (fig. 11) is complete.

CLEANING THE EXTRACTION CHAMBER

Between analyses for both carbon dioxide and oxygen the following is a quick and satisfactory method of cleaning either type of apparatus. The residual solution from the last analysis is ejected, either through the outlet tube of the upper cock or, preferably, into the cup, whence it is drawn off by suction. The level of the mercury is dropped to the bottom of the chamber by lowering the leveling bulb. Ten or 15 cc. of of water are admitted into the evacuated chamber. If carbon dioxide is to be determined in the next analysis, about 1 cc. of the normal lactic acid is also added. The chamber is shaken for a few seconds. The extracted gases and solution are then ejected. Such portions of the solution as remain adherent to the walls of the chamber are so nearly gas free that they cause no error in the subsequent analysis.

After use of the acid ferricyanide solution, as in determination of hemoglobin by the CO capacity method, some particles of methemoglobin are likely to cling to the walls of the chamber. These are quickly dissolved if a few drops of alkaline hyposulfite are added to the first portion of water used to rinse out the chamber after the analysis is finished.

Occasionally it is well to leave the chamber overnight full of chromic-sulfuric acid cleaning mixture. Chromic-sulfuric acid may be drawn down into the glass tubing below the chamber for a few minutes without injuring the rubber joint.

When not in use the chamber and cup of the apparatus are left filled with water.

LEAKAGE

It is advisable before beginning a series of analyses to test either the volumetric or manometric apparatus for leaks. Two or 3 cc. of water are introduced into the chamber and the air is extracted by shaking as described for oxygen determinations. The volume of extracted gas is read, in the case of the volumetric apparatus. In the manometric apparatus the manometer is read with the gas volume at 0.5 cc. The extraction is repeated. An increase in volume or pressure indicates a leakage of air into the evacuated apparatus.

The cause of a leak is usually improper grinding or lubrication of the upper cock. In the case of the volumetric apparatus, diffusion of air through the tube connecting the chamber with the mercury leveling bulb may let air enter the chamber from below, unless a Shohl trap is used (fig. 28). Diffusion through the tube of the manometric apparatus is prevented by the mercury seal (fig. 35), which makes a Shohl trap unnecessary.

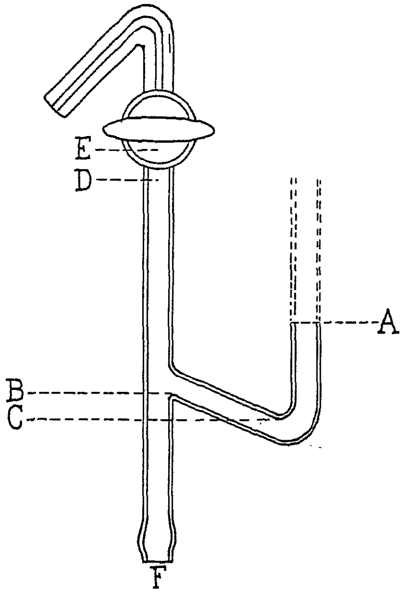


FIG. 28. Shohl air trap for volumetric apparatus. The trap is made of thick walled glass tubing and is sealed at *A* to the lower end of the apparatus shown in figure 26. The angle *B* must be less than 90 degrees, and *B* must be located higher than *C*. *E* is a cock from which one can occasionally eject air that diffuses through the rubber tube of the leveling bulb and collects in *D*. *F* connects with the mercury leveling bulb. From Shohl (34).

RUBBER TUBING

Diffusion through the rubber tube of the volumetric apparatus may be minimized by using a tube with very heavy walls and by keeping the leveling bulb routinely in position *2* (fig. 26). It should be lowered to position *3* only for the few seconds necessary to evacuate the chamber. Some tubing appears to be more porous than others, and the best tubing becomes porous after months of use. The best is the heavy-walled, greyish red, "nitrom-

eter tubing." If the above precautions fail to prevent significant leakage of air into the apparatus through diffusion, a glass air trap, such as that of Shohl (34) shown in figure 28, may be added to the apparatus. A Shohl trap is always desirable when the volumetric apparatus is to be used for O_2 or CO determinations.

LUBRICATION

All cocks must be well ground and must be so lubricated that they turn smoothly and do not leak. For lubrication successive application of vaseline and the vaseline-rubber mixture, described in chapter I has proven most successful.

When the cock at the top of the gas chamber in either the volumetric of manometric apparatus is to be lubricated, it is well to have some water in the chamber. After the cock is lubricated and replaced, this water is forced up into the cup. Then any small plug of grease that may have entered the capillary above or below the cock is forced up and out, instead of being drawn into the chamber, where it might adhere obstinately to the wall in the upper part.

Sometimes the curved capillary outlet at the top of the gas chamber may become so clogged with grease that the pressure obtained by raising the leveling bulb does not suffice to force fluid through. In this case the capillary can be cautiously warmed with the flame of a match or candle moved back and forth below it, or by wrapping it in a cloth soaked in hot water. The grease is softened, and can then be readily forced out.

USE OF CAPRYLIC ALCOHOL TO PREVENT FOAMING

With analyses of either whole blood or serum it is necessary to add caprylic alcohol to prevent foaming. It is desirable to keep the amount of caprylic alcohol small, 1 drop for plasma analyses and 2 drops for whole blood. The alcohol dissolves more air than does water, and may contain impurities with sufficient vapor tension to affect measurably the results yielded by the volumetric apparatus (the manometric is nearly immune to errors due to vapor tension). Each lot of caprylic alcohol should be tested by analysing standard carbonate solutions with and without addition of the alcohol. If the latter causes an appreciable error it should be redistilled under reduced pressure, the first portion of the distillate being rejected.

CALIBRATION OF THE CHAMBER OF THE APPARATUS

The chamber of either the volumetric or manometric apparatus is disconnected from the leveling bulb and is fastened upright in a clamp.

To the outlet at the bottom is attached a short capillary tube bearing a two-way cock, and drawn out to a fine point below. The attachment is made glass to glass with a piece of heavy walled "nitrometer" rubber tubing. By suction applied to the outlet capillary at the top of the chamber, the latter is filled with water drawn up from below. The lower cock is then closed and the upper cock turned so that the chamber is connected with the air in the cup. By manipulation of the lower cock water is then drawn off and weighed to within 1 mg. under paraffin oil, as described for calibration of burettes in chapter I.

The calibration device shown in figure 5 of chapter I can also be used, in place of the above technique.

The scale of the volumetric chamber is calibrated at 0.1-cc. intervals and a curve is made showing the volumes which must be added to or subtracted from the scale readings in order to give the true readings.

The manometric apparatus requires precise calibration at only two points the 0.5 and 2.0-marks (fig. 35). It is desirable to etch on the chamber the exact volumes measured at the 0.5 and 2.0-cc. marks.

HANDLING BLOOD SAMPLES

Blood for determination of oxygen or carbon dioxide must be drawn and handled before analysis without contact with air, except for the momentary exposure of a small surface that occurs when the sample is drawn into a pipette. If a large surface of the blood is exposed even momentarily enough oxygen may be taken up or carbon dioxide given off to affect measurably the results. Therefore blood for gas analyses must be drawn and handled by the anaerobic technique described in chapter II.

MEASURING SAMPLES OF BLOOD INTO THE GAS APPARATUS

*Types of pipettes used**

For delivery of 1 and 2-cc. samples of blood, pipettes of the type shown in figure 30 have been found most satisfactory. They are made with heavy walled capillary stems of 1-mm. bore, and are provided with stop-cocks, as shown in figure 30. They are calibrated to deliver between two marks, one on the upper stem and one above the cock on the lower stem. Each pipette is provided at the tip with a rubber ring, as shown in figures 29 and 30.

* An automatic pipette designed especially for use with the blood gas apparatus was described by Guest after this chapter had gone to press. The description is added to the appendix of this volume.

This ring is made by cutting a length of about 1 cm. from a tube of soft rubber, with a bore of 1 mm. and walls 2 mm. thick. The ring is tapered somewhat by grinding down the outer edge of one end with sandpaper or an emery wheel, at such an angle that the rubber will fit into the bottom of the

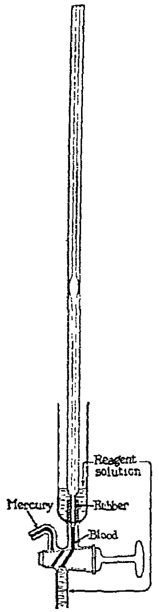


FIG. 29

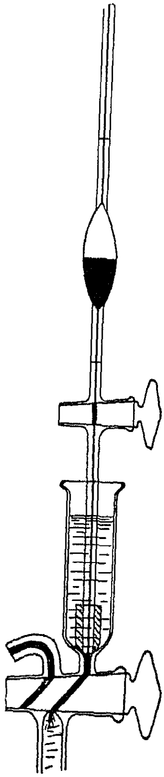


FIG. 30

FIG. 29. Delivery of blood sample into chamber of gas apparatus from rubber tipped pipette without stop-cock.

FIG. 30. Delivery of blood sample into chamber of apparatus from rubber tipped stop-cock pipette. From Van Slyke and Neill (59).

cup of the gas chamber, as shown in figure 29 and 30. A pipette equipped with such a rubber ring will deliver fluid directly into the chamber of the gas apparatus. The presence of the stop-cocks is not necessary for accuracy, but is a decided convenience.

For delivery of samples of 0.5 cc. or less pipettes calibrated for blow-out

delivery are generally used, as shown in figure 29. The 0.5-cc. pipette has a stem of 1 mm. bore. Pipettes of 0.2 capacity are made with stems of 0.6 or 0.7 mm. bore, and of the same shape as the pipette shown in figure 29. Pipettes of 0.1 cc. are made of straight capillary tubing of about 1 mm. bore. It is convenient to have 0.2 and 0.1-cc. pipettes calibrated both *to deliver* and *to contain*, as in some analyses they are used in the latter manner.

Sometimes when a sample of blood is drawn up into a pipette small bubbles rise to the top, so that exact measurement of the sample is difficult. In such a case the bubbles are dissipated if one touches them with a fine wire that has been dipped into caprylic alcohol.

Delivery from rubber tipped pipette without stop-cock (figure 29)[2]

This is the most economical, precise method of delivering samples of blood into the gas apparatus, for all the blood drawn into the pipette is utilized in the analysis. Hence the method is used in micro analyses, with samples of 0.5 cc. or less blood. It can be used equally well with larger samples, but when plenty of blood is available the stop-cock pipette is somewhat more convenient.

The pipette is filled above the mark on the upper stem with blood. It is then placed in a nearly horizontal position and the excess blood is drawn from the tip by absorption with a filter paper or towel, until the blood surface in the upper stem has fallen to the level of the mark. The pipette is then tilted a little from the horizontal, with the tip upwards, so that the surface of the blood in the stem rises about 2 mm. above the mark. The upper stem of the pipette is then closed with a finger, and pipette tip is pressed into the bottom of the cup of the gas apparatus as shown in figure 29. When the pipette is changed from the horizontal to the vertical position the surface of the blood in the stem should move down the stem just far enough to return to the calibration mark.

Before the pipette tip is inserted into the cup of the gas apparatus the latter is arranged as shown in figure 29, with the cock there shown open, and the cock (not shown) leading to the mercury leveling bulb closed. The reagents with which the blood is to be mixed in the chamber are, as shown in figure 29, to be partly in the chamber and partly in the cup.

After the pipette tip has been pressed into position in the cup the finger is removed from the opening at the top of the pipette, and the

[2] This procedure has not been previously described. It was developed, after the original papers had been published, in order to meet a situation which required precise and economical delivery of 0.5 cc. blood samples.—D. D. V. S.

flow of blood into the chamber is regulated by cautious opening of the *cock which leads to the mercury leveling bulb.* It is possible to regulate the flow of mercury through a cock more smoothly than the flow of blood: hence the mercury cock is used instead of the cock shown in figure 29. During the admission of the blood the mercury leveling bulb is at such a level as to cause a slight negative pressure in the chamber of the gas apparatus.

The delivery is continued until the blood has entirely left the pipette, and a bubble of air has followed the column of blood into the capillary beneath the cup of the gas apparatus, as shown in figure 29. At this moment the cock shown in figure 29 is closed. The pipette is then withdrawn from the cup. The bubble of air left in the capillary is dislodged by means of a fine wire that has been dipped into caprylic alcohol. As much of the reagent solution or water from the cup as is desired is then admitted into the chamber, washing in with it the blood in the capillary of the cock.

Delivery from rubber tipped pipette with stop-cock

If a pipette with a stopcock is used, the procedure is similar, except that at the beginning it is not necessary to make the blood in the pipette flow above the mark in the upper stem. The blood sample is drawn up to the mark, and the cock is closed. The pipette is then placed in position as shown in figure 30, and the flow of blood is regulated as above described by manipulation of the cock leading to the mercury bulb. The flow of blood in this case is stopped when the blood surface in the pipette has fallen to the mark on the lower stem: at this moment the cock of the pipette is closed and likewise the cock of the gas apparatus shown in figure 30. The pipette is withdrawn from the cup, and as much of the reagent solution in the cup as is desired is admitted into the chamber, washing in with it the blood in the capillary.

Delivery from pipette without either rubber tip or stop-cock

The pipette tip is rested on the bottom of the cup during delivery, the cup being partially filled with water or reagent solution, as in the above cases. As the blood flows out from the tip of the pipette the cock at the top of the chamber of the gas apparatus is opened enough so that fluid flows into the chamber a little faster than blood flows out of the pipette. The blood therefore flows continually into the chamber with a stream of water or reagent solution from the cup, which washes

in the blood as rapidly as it emerges from the pipette tip. This method of delivery was the one first used in developing the technique of blood gas analysis described in this chapter, and is capable of precise results. It requires, however, more skill and is more liable to mischance than the two procedures described above, in which the tip of the pipette is provided with a rubber ring.

With any type of pipette it is essential for accuracy that the delivery be at a rate so slow that uniform drainage from the pipette walls is obtained.

ADMISSION OF ABSORBENT SOLUTIONS INTO THE CHAMBER

Sodium hydroxide, sodium hyposulfite, and cuprous chloride solutions are added directly into the chamber to absorb carbon dioxide, oxygen and carbon monoxide, respectively. After adding any of these solutions through the upper cock, a drop of about 0.01 cc. usually adheres to the lower side of the cock, at the top of the calibrated stem of the apparatus. This fluid if left would cause an error in the reading of the volume or pressure of gas. *The drop is readily detached, however, by running a little mercury through the cock. This should be done as a routine after any absorbent solution is admitted into the chamber of either the manometric or volumetric apparatus.*

After an absorbent solution has been added through the top of the chamber about one minute is required for it to *drain completely* down the walls of the space in which the gas volume is measured. One must consequently *wait about one minute before reading the gas volume or the manometer* in the volumetric or manometric types of apparatus respectively. Otherwise the amount of residual gas will appear to be measurably greater than it really is. For example, in oxygen determinations in the manometric apparatus, if the pressure of $O_2 + N_2$ on the manometer is read immediately after the CO_2 is absorbed with 1 N sodium hydroxide, the pressure observed will be as a rule about 1 millimeter greater than it is when read a minute later, after drainage has been completed. The reason is that when the immediate reading is taken the gas volume above the 2.000-cc. mark is really a little less than 2 cc. because of the small volume of alkali solution which has not yet drained down from the walls.

READING MENISCUS OF SOLUTION IN CHAMBER

Both types of chambers are calibrated to give correct results when the *bottom of the water meniscus is read.* The bottom of the meniscus, in the tubes of about 4-mm. bore, is approximately 1.5 mm. below the top, so that

a marked error would be made if the top were read. With deeply colored solutions, such as those of whole blood, it requires some practice to judge accurately where the bottom of the meniscus is, and there is a tendency at first to read a point about 0.5 mm. too high. *If a "frosted" light is placed about 50 cm. behind the chamber* when such solutions are in it the exact location of the meniscus bottom is defined. Such a light is almost indispensable for exact analyses of whole blood.

SEALING THE COCK OF THE CHAMBER WITH MERCURY

An ordinary glass cock will not hold a vacuum unless the bore of the cock is filled with mercury. In using either the manometric or the volumetric apparatus the curved capillary outlet at the left of the cock of the chamber is kept continually filled with mercury, likewise the bore leading through the cock to this capillary. The other bore, leading to the cup above the chamber, must be filled with mercury each time before the chamber is evacuated. The sealing of this cock with mercury is carried out as follows:

After blood or reagent solutions have been measured into the chamber, and before the chamber is evacuated, the cup at the top is routinely about half filled with water, and about 0.2 cc. of mercury is dropped in. The mercury is admitted into the chamber until just enough is left above the cock to fill the capillary leading up to the cup (see figure 35). The addition of the water precedes that of the mercury, because if the mercury were dropped into the empty cup it would be difficult to avoid trapping air in the capillary with the mercury. After the cock has been sealed with mercury the water is removed from the cup, and the next step of the analysis is begun.

ANALYSES WITH THE VOLUMETRIC APPARATUS

READING GAS VOLUMES AT ATMOSPHERIC PRESSURE IN THE VOLUMETRIC APPARATUS

After the gases have been extracted from solution in each analysis the solution in the chamber is drained off into the bulb below as completely as possible before mercury is readmitted and the reading is taken. However, a slight film of water always remains on the walls of the chamber and collects to the extent of a few hundredths of a cubic centimeter on the surface of the mercury as the latter rises through the chamber. Consequently exact atmospheric pressure is not obtained by placing the surface of the mercury in the leveling bulb opposite that in the chamber. The mercury in the leveling bulb must be slightly

higher than that in the chamber in order to balance the column of water on top of the latter. The level is obtained with sufficient accuracy by estimating with the eye the height of the water column (fig. 31) and raising the mercury meniscus in the bulb 1 mm. above that in the chamber for every 13 mm. depth of the column of water solution. As

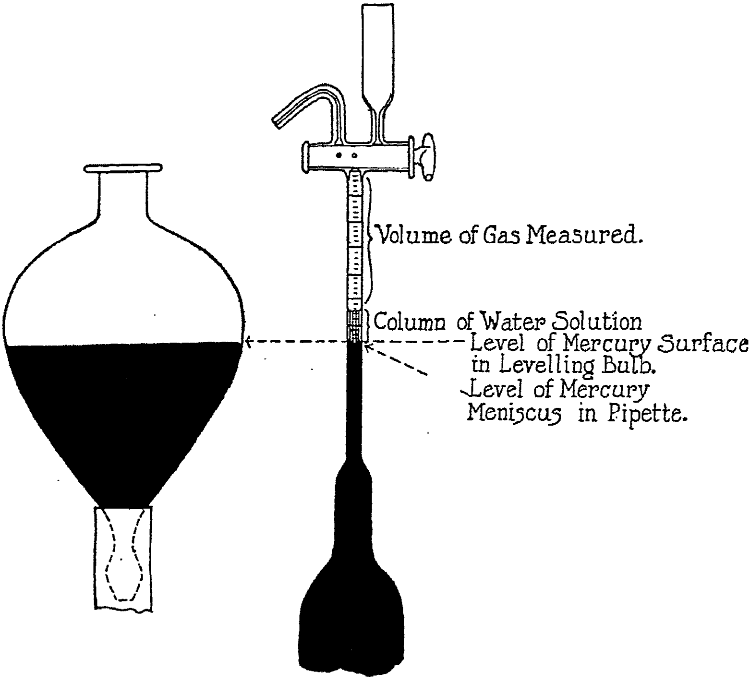


FIG. 31. Conditions for reading gas volume at atmospheric pressure in the volumetric blood gas apparatus. From Van Slyke (44).

the latter is usually not more than equivalent to 1 mm. of mercury, the correction is insignificant.

CARBON DIOXIDE IN PLASMA OR WHOLE BLOOD[3]

Reagents

0.1 N lactic acid, 1 N NaOH, and caprylic alcohol, described previously under "General reagents."

[3] The use of the apparatus originally described by Van Slyke (44, 45) is somewhat modified by improvements in reagents and manipulations later introduced by Van Slyke and Stadie (64) and Van Slyke and Neill (59).

Procedure

After having been cleaned and tested for leaks as described above, the apparatus (fig. 26), including both capillaries above the upper cock,

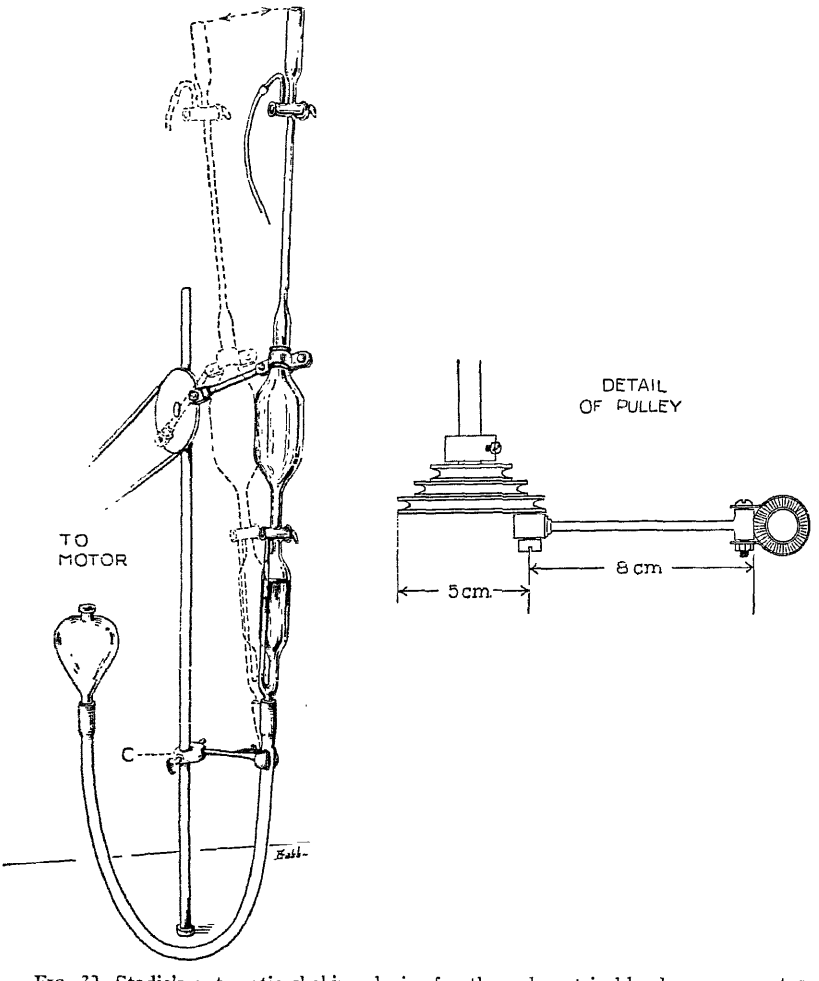


FIG. 32. Stadie's automatic shaking device for the volumetric blood gas apparatus It is run at a speed of 200 to 250 revolutions per minute. From Stadie (38).

is filled with mercury. One drop of caprylic alcohol is placed in the bottom of the cup and permitted to flow down into and fill the capillary beneath. Two cubic centimeters of carbon dioxide-free 0.1 N lactic

acid are placed in the cup, and the sample of 1 cc. of blood or plasma is run into the chamber underneath this aqueous layer, as described above under "Measuring samples" and shown in figure 29 or 30. Sufficient of the acid is admitted after the sample to bring the volume of solution in the apparatus to 2.5 cc. A drop of mercury is then placed in the cup; it is permitted to fill the capillary and bore of cock *e*, to act as seal to the latter, and the cock is closed. During the above operations the mercury bulb is at position 2 (fig. 26) and the lower cock is open, so that slight negative pressure is maintained in the chamber.

In order to extract the carbon dioxide from solution the mercury bulb is lowered to position 3 (fig. 26) and held there until the meniscus of the mercury (not of the solution) in the chamber has fallen to the 50-cc. mark. The lower cock is then closed and the leveling bulb is returned to position *2*. The carbon dioxide is now extracted from the solution by shaking the latter either by hand, or at the rate of about 200 revolutions per minute with the Stadie shaker (fig. 32). For shaking the apparatus by hand in plasma analyses the chamber is removed from its holder and inverted 15 or more times. With whole blood it is preferable to rotate the solution about the walls of the chamber in order to avoid foam. With the Stadie shaker two minutes agitation is used. The chamber is then returned to its holder and allowed to stand for a minute to permit the solution to drain down from the walls to the bottom. The leveling bulb is once more lowered to position *3*, the lower cock is opened to connect the chamber with trap ***d*** under the lower cock, and the fluid from the chamber is drained down into the trap, care being taken that no gas is permitted to follow. The leveling bulb is raised to approximately the position shown in figure 31. The lower cock is then turned so that mercury is admitted to the chamber through tube ***c*** (fig. 26) until the gas is brought to atmospheric pressure as shown in figure 31. The stop-cock is not opened suddenly so that the mercury swirls when it first enters the chamber, but is gradually opened while the mercury is rising through the expanded portion of the chamber. During the admission of the last part of the mercury the flow is retarded by partially closing the lower cock, so that the meniscus in the calibrated stem comes slowly and smoothly to its position of equilibrium with the atmospheric pressure, as shown in figure 31. The lower cock is then closed and the gas volume is noted. If the layer of solution floating on the mercury is permitted to oscillate up and down it will reabsorb enough carbon dicxide to cause appreciable error in the determination.

In the case of plasma the reading of the total gas volume may be taken as the finish of the determination. A certain amount of air carried into the apparatus dissolved in the plasma and 0.1 N lactic acid is mixed with the carbon dioxide, but the correction necessary for this admixed air may be calculated from the solubility of air in water at room temperature and in plasma at 38° with sufficient accuracy for most purposes. The air corrections so calculated are given in the second column of table 24.

When CO_2 in plasma is thus determined, without the introduction of alkali, it is not necessary to wash the apparatus out between analyses. The slight amount of CO_2 remaining dissolved in the film of acidified solution, which adheres to the chamber walls after the reagents are ejected, is negligible.

If the analysis is thus shortened by omitting alkali absorption of CO_2 the apparatus must be provided with a Shohl trap (fig. 28), or its equivalent, to prevent the slightest access of air to the chamber during the analysis. A blank extraction of 2.5 cc. of the 0.1 N lactic acid should yield no more extracted gas than the volume indicated in the second column of table 24.

If whole blood is analyzed so much oxygen is mixed with the carbon dioxide that it is necessary to determine the latter always by absorption with alkali. The gases are first extracted and their volume measured as above described. Then the leveling bulb is lowered so that a partial vacuum is obtained and the gas space is increased to about 5 cc. in the chamber. The lower cock is then closed and 2 cc. of the gas-free 1 N sodium hydroxide solution is placed in the cup of the apparatus. 1 cc. of the alkali is allowed to flow slowly into the chamber, at least 30 seconds being taken for its admission. As it flows down the walls it completely absorbs the carbon dioxide. If the alkali solution were added at atmospheric pressure it would absorb along with the carbon dioxide a slight amount of the oxygen. After the alkali has been admitted the upper cock is sealed with mercury, of which a few drops are permitted to flow through the cock to dislodge the drop of alkali usually adhering to the lower side of the cock. The leveling bulb is now lowered to position 3 and the alkali solution is drawn down into the 15-cc. bulb with the other solutions previously trapped there. The volume of unabsorbed oxygen and nitrogen is then measured. The difference between this and the first reading represents the volume of carbon dioxide.

This procedure is also used in analyses of plasma when maximum accuracy is desired.

Calculation of carbon dioxide content. When the carbon dioxide is extracted in the evacuated apparatus about 5 per cent of the total remains in the water solution. This residue is left in solution because carbon dioxide is soluble in water to such an extent that, when equilibrium is attained at room temperature between the water and the gas phase, carbon dioxide divides itself between the two phases in about equal concentrations, 1 cc. of the water containing nearly the same amount of carbon dioxide as 1 cc. of the gas space. Since under the conditions of analysis the water solution occupies 5 per cent of the volume of the 50 cc. chamber during the extraction, approximately 5 per cent of the carbon dioxide remains in the solution when extraction has become as complete as possible. The exact amount varies with the temperature, since carbon dioxide is less soluble in warm than in cold water. The solubility of CO_2 is also affected by the presence of other dissolved substances, but this effect is negligible for the present analysis. The entire calculation was expressed by Van Slyke and Stadie (64) in the equation

$$(1) \qquad V_{O^\circ,760} = i\,V_t \underbrace{\frac{(B - W)}{760\,(1 + 0.00367\,t)}}_{\text{Factor correcting for atmospheric pressure and temperature.}} \times \Big(1 + \underset{\text{Factor correcting for unextracted carbon dioxide.}}{A - S}$$

In this formula, $V_{O^\circ,760}$ represents the carbon dioxide content of the analyzed solution in cubic centimeters of the gas measured at 0°, 760 mm.; V_t represents the observed volume of carbon dioxide measured in the apparatus at room temperature, t°, in the analysis carried out as above described; B is the barometric pressure; W is the vapor pressure of the water solution at t°; S is the volume of water solution in the apparatus; A is the total volume of the extraction chamber, viz., 50 cc.; and α' is the ratio in which carbon dioxide distributes itself between equal volumes of the aqueous and gaseous phases when extraction is completed. The values of α' for CO_2 are given in table 27.

There is one other correction included in the above formula besides those for pressure, temperature, and solubility of the gas in water. While the volume of the gas phase is being reduced from 47.5 cc. to its volume at atmospheric pressure (usually about 0.6 cc.) a small proportion of the carbon dioxide is reabsorbed from the gas phase into the water. Under the conditions of the analysis Van Slyke and Stadie (64) found that this reabsorption averaged 1.7 per cent of the extracted carbon dioxide. Con-

TABLE 24

FACTORS FOR CALCULATING CO_2 CONTENT DETERMINED BY VOLUMETRIC APPARATUS ..... BLOOD OR PLASMA SAMPLES OF 1 CC. (FROM VAN SLYKE AND STADIE (64))

TEMPERATURE	AIR IN EXTRACTED GASES FROM PLASMA AND WATER. SUBTRACT FROM OBSERVED AIR + CO_2 VOLUME IF CO_2 AND AIR ARE MEASURED TOGETHER	FACTORS BY WHICH CUBIC CENTIMETERS OF CO_2 EXTRACTED FROM 1 CC. PLASMA OR BLOOD ARE MULTIPLIED TO GIVE	
		Volume per cent CO_2	Millimoles CO_2 per liter
°C.	cc.		
15	0.048	$100.2 \times \frac{B}{760}$	$44.9 \times \frac{B}{760}$
16	48	99.5 "	44.7 "
17	48	98.9 "	44.4 "
18	47	98.3 "	44.2 "
19	47	97.8 "	43.9 "
20	46	97.2 "	43.7 "
21	46	96.6 "	43.4 "
22	45	96.0 "	43.1 "
23	45	95.4 "	42.9 "
24	45	94.8 "	42.6 "
25	44	94.2 "	43.3 "
26	44	93.6 "	42.1 "
27	44	93.1 "	41.8 "
28	43	92.4 "	41.5 "
29	43	91.8 "	41.3 "
30	43	91.2 "	41.0 "
31	43	90.6 "	40.7 "
32	42	90.0 "	40.4 "
33	42	89.4 "	40.2 "
34	42	88.8 "	39.9 "

BAROMETER	$\frac{\text{Barometer}}{760}$	BAROMETER	$\frac{\text{Barometer}}{760}$
732	0.961	756	0.995
734	0.996	758	0.997
736	0.967	760	1.000
738	0.971	762	1.003
740	0.974	764	1.006
742	0.976	766	1.008
744	0.979	768	1.011
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

sequently the results calculated by the above equation must be multiplied by an empirical factor, indicated as i in the formula, which has an average value of 1.017.

The factors calculated by the equation are given in table 24. When the carbon dioxide volume in the apparatus is determined by absorption with sodium hydroxide it is multiplied directly by the factor indicated. When, as may be done in plasma analyses, only one reading is made, that of the total carbon dioxide plus air, the estimated cubic centimeters of air held in solution under room conditions by 1.5 cc. of water plus 1 cc. of plasma (second column of table 24) are subtracted from the observed gas volume, and the remainder is multiplied by the carbon dioxide factor (third or fourth column of table 24) corresponding to the observed temperature.

PLASMA CARBON DIOXIDE COMBINING CAPACITY. VAN SLYKE AND CULLEN (53)

In this method the blood is drawn with limited exposure to air and is immediately centrifuged. Before the analysis the plasma is equilibrated at room temperature with air containing 5.5 per cent of CO_2, in order to restore CO_2 which has escaped from the plasma during and after centrifugation. In calculating the results the physically dissolved CO_2 is subtracted, so that the value obtained represents CO_2 in the form of bicarbonate.

This method was introduced in 1917 to measure the marked fall in plasma bicarbonate content that occurs in the acidoses of diabetes, nephritis, and other conditions in which the bicarbonate fall is due to displacement by the anions of other acids. The method proved definitely practical, and is still in use in many clinical laboratories. It does not give so exact an indication of the state of the acid-base balance in the blood as does direct determination of the CO_2 content made on plasma obtained under complete anaerobic precautions, and without equilibrating with 5.5 per cent CO_2. The equilibration with air of physiologically normal CO_2 tension at room temperature gives the plasma a content of H_2CO_3 considerably above the level of physiological normality because the solubility of CO_2 at 20° is 1.6 times as great as at 38°. In consequence, the equilibrium, H_2CO_3 + B Protein = $BHCO_3$ + H Protein, is shifted to the right so that $BHCO_3$ is increased also. The bicarbonate content, or "CO_2 capacity" found in venous or arterial blood plasma by this procedure, in blood handled strictly without contact with air, was found in fact by Stadie and Van Slyke (38a) to be about 3 volumes per cent higher than the total CO_2 content of the plasma analyzed immediately after it was centrifuged. The relationship is quite constant, so that for the purpose of following pathological changes in the alkali reserve in

the conditions above mentioned, the CO_2 capacity is entirely serviceable. In the conditions where it is doubtful whether over or under ventilation of CO_2, rather than loss or retention of non-volatile acids, is responsible for an acid-base disturbance, it is necessary to determine both the pH and the CO_2 content in plasma as nearly as possible unchanged from its condition in the circulation; and in these cases the Van Slyke-Cullen CO_2 capacity method can not be used in place of direct CO_2 analysis of the anaerobically separated plasma. Such conditions form a small part of those in which knowledge of the alkali reserve is of clinical importance, however. In the majority of cases the physician desires to know whether a diabetic or nephritic is threatened with acid intoxication, or whether vomiting or alkali therapy has produced a state of alkali excess in the blood. In these conditions the changes in plasma bicarbonate are gross ones (see table 68 on page 1001 of volume I), the primary disturbance is in the alkali reserve, and the CO_2 capacity method gives results practically as valid as CO_2 determinations on anaerobically separated plasma.

The practical advantages of the CO_2 capacity method are that the blood for it can be drawn without strict anaerobic precautions, can be centrifuged without protection by oil or other special precautions, and the plasma after separation can be kept in an open tube for several hours before it is finally resaturated with CO_2 and analyzed.

When equipment for anaerobic handling of blood is at hand these advantages are not important. It is then practically as convenient to handle all blood anaerobically as a routine, and determine the CO_2 content in the plasma or serum thus obtained. This in fact is the practice in the laboratories of both the authors, who have for some years, since the anaerobic technique was perfected, abandoned the use of the original plasma CO_2 capacity method. When anaerobic blood handling equipment is not at hand, however, or is insufficient to be used with all bloods studied, or the physicians who draw the blood are not familiar with its use, the capacity method still fills a definite place, and for this reason is retained in the present volume.

Reagents

Same as for CO_2 determination, above.

Procedure

Drawing blood sample. The blood, preferably 5 cc. or more, may be drawn into an oxalated centrifuge tube arranged as shown in figure 7, I of chapter II. The layer of oil in this case is not necessary. Or the

blood may be drawn into a syringe and transferred to a tube. From the syringe the transfer to a centrifuge tube should be made with the tip of the needle at the bottom of the tube so that the blood does not fall through the air. The blood in the tube is stirred gently just enough to mix it with the oxalate dissolved from the wall of the tube. The blood is subject to no other agitation or the loss of CO_2 may be sufficient to cause such a transfer of Cl from cells to plasma that the alkali reserve of the latter is significantly diminished.

Centrifugation. Within a few minutes after the blood has been drawn it should be centrifuged. An open tube may be used, without protection to prevent loss of CO_2 from the fluid. Some CO_2 is lost from the upper layer of the plasma, but the lower layers in contact with cells are so little affected in the time required for centrifuging that the

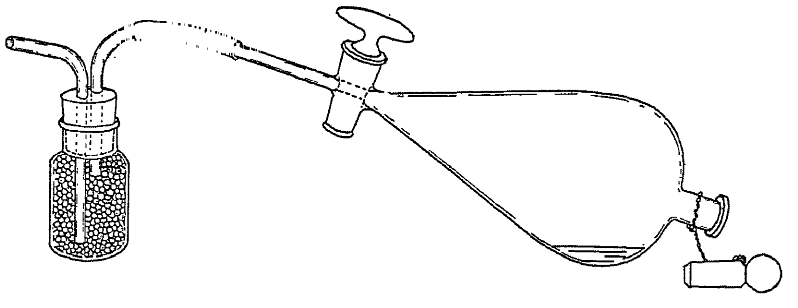


FIG. 33. Separatory funnel arranged for saturating plasma with alveolar air. From Van Slyke and Cullen (53).

electrolyte exchange between cells and plasma is not sufficient to alter significantly the bicarbonate content of the latter.

Storage of separated plasma for analysis. Immediately after centrifugation the plasma is pipetted off from the cells and transferred to a clean test tube. Its CO_2 capacity will there remain unchanged for the duration of a working day. If the tube, before the plasma is transferred to it, is provided with a paraffin lining, and is stoppered and placed in a refrigerator as soon as the plasma has been placed in it, the CO_2 capacity of the latter will remain unchanged for several days. If the plasma is left in contact with ordinary glass for more than a few hours enough alkali may be dissolved to increase measurably the CO_2 capacity.

Saturation of the plasma with air containing approximately 5.5 per cent of CO_2. As a saturating vessel a 300-cc. separatory funnel

of the type shown in figure 33 is convenient. To obtain material for duplicate macro analyses, 3 or 4 cc. of plasma are placed in the funnel. The latter is then turned on its side, and the air in it is replaced either by alveolar air from the lungs of the operator or by a 5.5 per cent CO_2-air mixture from a tank. In either case the mixture must be passed over glass beads before it enters the funnel (see fig. 33). Otherwise when air from the lungs is used the plasma is appreciably diluted with moisture which condenses from the breath on the inner walls of the funnel.

By passage over a large surface of either wet or dry glass beads at room temperature the expired air is cooled, and the excess moisture in it is condensed, so that not enough is carried into the funnel to cause an appreciable error. When, on the contrary, a dry CO_2-air mixture from a tank is used, it causes an appreciable evaporation from the surface of the plasma, with consequent increase in its concentration and in the carbon dioxide capacity. This also is obviated if the gas mixture is passed over wet beads, so that it approaches saturation with water vapor.

For obtaining an artificial mixture of air containing 5.5 per cent of CO_2 one may use an ordinary metallic gas tank capable of standing 20 atmospheres pressure and provided with an accurate pressure gage. Carbon dioxide is run in from another tank until the desired pressure is indicated. Then air is run in until the total pressure of air plus CO_2 is 18.2 times that of the CO_2 (taking into account that the tank contains one atmosphere more than the gage registers). The tank is then laid on its side for a half-hour to give the gases an opportunity to mix thoroughly, and samples are drawn for analysis before the mixture was used. The analysis has to be repeated every few days, as the CO_2 content of the gas sometimes changes. In order to displace completely the air in the separatory funnel with the CO_2 mixture, five or more volumes are run through, the gas, after leaving the funnel, being collected in a gasometer or rubber bag so that the volume passed can be roughly estimated.

When alveolar air is used, the operator, without inspiring more deeply than normal, expires as quickly and as completely as possible through the bottle of glass beads and the separatory funnel connected as shown in figure 33. The stopper is inserted just before the expiration is finished, so that there is no opportunity for outside air to be drawn back into the funnel. With a little practice a normal person can consistently fill a 300-cc. separatory funnel with air containing within a few tenths of a per cent of the desired 5.5 per cent of CO_2. The composition is not, of course, so constant as that obtained when an analyzed gas mixture is used to fill the funnel, but as a matter of experience we have never found that the deviations caused significant error in the results.

A change of 0.5 per cent in the CO_2 concentration of the air with which the plasma is shaken causes a change of only about 1 volume per cent in the CO_2 plasma CO_2 content, which is normally 60 to 80 volumes per cent.

In order to saturate the plasma the funnel is rotated for two minutes so that the plasma is distributed in a thin layer as completely over the interior of the funnel as is possible. Two minutes rotation in this manner has been uniformly found to be sufficient, but one minute is usually not enough. When several analyses are to be done they can be performed at one time by placing the funnels in a rack which can be turned, or by binding them together with elastic bands. The Stadie rotator, shown in figure 34, can be used.

Determination of carbon dioxide content of the saturated plasma. The stoppered funnel is placed upright for a few minutes, so that the plasma drains to the contracted lower end. One cubic centimeter portions of the plasma are drawn up into pipettes calibrated for complete delivery, and the analyses are carried out as described previously for CO_2 determinations on plasma. The delivery of the 1 cc. samples into the chamber of the apparatus is accomplished by the technique shown in figure 29 on page 240. The total volume of CO_2 + air extracted from the 2.5 cc. of acidified plasma solution is measured. The analysis is then finished. Absorption of the CO_2 with alkali is unnecessary, since the correction for the amount of air present can be calculated with sufficient accuracy from the solubility of air in water.

Because no alkali is used, the apparatus can be used for a series of determinations without washing it out between analyses. The film of acid solution left on the wall after the solution analyzed has been ejected does not contain enough CO_2 to affect the next analysis.

Calculation

Table 25 enables one to convert the observed gas volume readings into volumes per cent of CO_2 bound as bicarbonate directly, without calculation except that required to correct for barometric pressure. It is usually sufficiently exact if one makes the estimation to the nearest volume per cent.

Example. The observed gas volume is 0.78 cc. at 23°, 752 mm. Multiplying by 752/760, or 0.989 (bottom of table 24) reduces the gas volume to 0.774 cc. Reference to table 25 shows that at 23° this indicates 65 volumes per cent of CO_2 binding capacity.

The figures in table 25 were calculated by Van Slyke and Cullen by subtracting from the observed volume of extracted air and CO_2 a correction for the volume of air dissolved at room temperature in the 2.5 cc. of solution, and for the volume of CO_2 physically dis-

TABLE 25

For Calculation of the Carbon Dioxide Combining Capacity of Blood Plasma (from Van Slyke and Cullen (53))

Observed vol. gas $\times \frac{B}{760}$	Cubic centimeters of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 cc. of plasma				Observed vol. gas $\times \frac{B}{760}$	Cubic centimeters of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 cc. of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.6	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

solved as H_2CO_3 when the plasma was saturated with air containing 5.5 per cent of CO_2. This correction at ordinary temperatures is about 0.05 cc., nearly the same as the correction for dissolved air. The corrected gas volume, representing CO_2 from $BHCO_3$, is then reduced to 0°, 760 mm. by the usual factors. In addition a correction is made for the effect of variations in temperature at the time of saturation on the amount of CO_2 bound as bicarbonate. Between 10° and 30° it was found that 1° rise of temperature at the time of saturation decreased the amount of bicarbonate CO_2 by an average of 0.36 per cent of its value. A correction for this effect is also included in computing table 25. The figures in table 25 are so calculated that, regardless of the room temperature at which saturation and analysis are performed, the carbon dioxide combining power indicated by the table represents the volumes per cent of CO_2 that the plasma can bind as bicarbonate when saturated at 20° with air containing CO_2 at approximately 41 mm. tension. If the results so obtained are multiplied by 0.94 they indicate within 1 or 2 per cent the amount of bicarbonate in plasma saturated at the same CO_2 tension but at body temperature (37–38°). It has been the convention to use the values for 20°.

OXYGEN IN BLOOD

The oxygen combined with hemoglobin is set free by ferricyanide, which changes the hemoglobin to methemoglobin. The freed oxygen gas, together with some carbon dioxide, is extracted. The CO_2 is absorbed with alkali. The O_2 is then measured by absorption with sodium hyposulfite solution.

Reagents

Neutral ferricyanide-saponin solution.
1 N sodium hydroxide solution, gas-free.
Alkaline hyposulfite solution, gas-free.
Caprylic alcohol.
These have all been described in this chapter under "General reagents."

Procedure

A drop of caprylic alcohol and 10 cc. of the ferricyanide solution are run into the chamber of the apparatus (fig. 26), the upper cock is sealed and the chamber is evacuated by lowering the leveling bulb and opening the lower cock. *All* the mercury is run out of the chamber, leaving only the solution in it. The solution is now shaken thoroughly in the evacuated chamber to extract the air. If during this procedure mercury were left in the chamber it would decompose part of the ferricyanide. In case the Stadie shaker is used, three minutes are taken for the extraction of the air. If the extraction is done by hand the time required is usually shorter, but depends somewhat on the way in which the manipulator handles the apparatus, which is shaken in such a manner that the solution is whirled about the walls. After extraction

is complete the chamber is returned to its holder and mercury is readmitted. The extracted air is ejected through the cup and 6 cc. of the solution are forced up into the cup. It is advisable before this moment to have the 2 cc. blood sample ready in a stop-cock pipette. If the Stadie shaker is used one may employ the three minutes required for extraction of air from the reagent solution to mix the blood and draw a sample into the pipette shown in figure 30. As quickly as possible after the 6 cc. of reagents have been forced up into the cup, the blood sample is run into the chamber of the apparatus, as described above under "Measuring samples." One cubic centimeter of the reagent solution is run in after the blood, leaving 5 cc. in the cup. This portion left in the cup is discarded. Its purpose has been to serve as a protecting layer to prevent diffusion of atmospheric air into the 1 cc. at the bottom, which is used to wash the last drops of blood into the chamber.

The sample and reagents being in the chamber, the upper cock is sealed with a drop of mercury. The apparatus is now evacuated, the mercury is withdrawn from the chamber, and the latter is shaken two to three minutes to extract the oxygen set free by the ferricyanide from combination with the hemoglobin. If the shaking is done by hand it is preferable to whirl the solution about the walls of the chamber, rather than to invert the apparatus repeatedly. Whole blood tends to foam despite the caprylic alcohol if the chamber is shaken by inversion.

When extraction is complete the gas volume is reduced to about 5 cc. and 2 cc. of the gas-free 1 N sodium hydroxide solution are placed in the cup of the apparatus. One cubic centimeter of the alkali solution is run slowly into the partially evacuated space to absorb the carbon doxide which has been extracted from the blood with the oxygen. If the alkali solution were added to the oxygen-carbon dioxide mixture at atmospheric pressure it would absorb a slight amount of the oxygen along with the carbon dioxide. A few drops of mercury are then admitted from the cup to dislodge the drop of alkali that usually adheres under the cock at the top of the chamber.

The solution is now drawn down into the trap bulb below the chamber, and the oxygen and nitrogen gas extracted from the blood are measured as illustrated in figure 31. A "frosted" light about 50 cm. behind the chamber will usually be needed to show sharply the meniscus of the dark methemoglobin solution.

The oxygen is now absorbed as follows. Two cubic centimeters of

air-free hyposulfite solution are placed in the cup. The lower cock of the apparatus is opened, with the mercury bulb in position *2* (fig. 26)

TABLE 26

FACTORS FOR CALCULATING O_2, N_2, OR CO CONTENT DETERMINED BY VOLUMETRIC APPARATUS WITH BLOOD SAMPLES OF 2 CC.

TEMPERATURE	FACTORS BY WHICH CUBIC CENTIMETERS OF GAS EXTRACTED FROM 2 CC. BLOOD ARE MULTIPLIED TO GIVE	
	Volume per cent gas in blood	Millimoles gas per liter blood
°C.		
15	$46.8 \times \frac{B}{760}$	$20.9 \times \frac{B^*}{760}$
16	46.6 "	20.8 "
17	46.4 "	20.7 "
18	46.2 "	20.6 "
19	46.0 "	20.5 "
20	45.7 "	20.4 "
21	45.5 "	20.3 "
22	45.3 "	20.2 "
23	45.0 "	20.1 "
24	44.8 "	20.0 "
25	44.6 "	19.9 "
26	44.3 "	19.8 "
27	44.1 "	19.7 "
28	43.8 "	19.6 "
29	43.6 "	19.5 "
30	43.3 "	19.3 "
31	43.1 "	19.2 "
32	42.8 "	19.1 "
33	42.6 "	19.0 "
34	42.3 "	18.9 "

* When, in analyses of drawn blood, O_2 and N_2 or CO and N_2 are measured together, 1.2 volume per cent N_2 may be subtracted to estimate the O_2 or CO.

To estimate chemically combined O_2 in drawn or aerated blood, subtract from the total O_2 or $O_2 + N_2$ the proper correction for dissolved O_2 and N_2 in table 31. From O_2 content of aerated blood determined as a measure of *hemoglobin*, subtract 0.5 volume per cent from total O_2, or 1.9 volume per cent from $O_2 + N_2$, to estimate HbO_2.

so that the gas in the chamber is under slight negative pressure. The hyposulfite is admitted in small droplets at the rate of about 1 every five or ten seconds, so that a thin film is kept flowing continuously down

the chamber wall, until there is no further shrinkage in the gas volume. Usually about 0.5 cc. of hyposulfite solution suffices. The residual gas is reduced to a small bubble of 0.02 to 0.03 cc. of nitrogen gas. Another 0.5 cc. of hyposulfite solution is then run over this bubble. A few drops of mercury are next run through the cock to detach the drop

TABLE 27

DATA FOR CALCULATION OF FACTORS FOR MANOMETRIC BLOOD GAS APPARATUS (FROM VAN SLYKE AND NEILL (59))

TEMPERATURE	$\frac{1}{1+0.00384t}$	α'_{CO_2}	α'_{O_2}	α'_{CO}	α'_{N_2}
°C.					
15	0.9455	1.075	0.0365	0.0268	0.0177
16	21	43	59	64	75
17	0.9387	15	53	60	72
18	53	0.989	48	56	70
19	20	66	43	53	68
20	0.9286	42	37	49	65
21	55	19	32	46	64
22	21	0.896	26	43	62
23	0.9188	73	22	40	59
24	56	50	17	36	58
25	24	28	13	34	56
26	0.9092	08	09	31	54
27	60	0.789	05	28	53
28	29	72	00	26	51
29	0.8998	55	0.0295	24	50
30	67	38	90	22	49
31	36	24	87	19	47
32	06	10	83	16	45
33	0.8875	0.696	79	13	44
34	45	82	75	10	42

The α' values are obtained by multiplying the α values by $1 + 0.00367\ t$. The α values are from Landolt-Börnstein's tables, the figures taken for CO_2 being those of Bohr and Bock, for O_2 the mean of those of Bohr and Bock and of Winkler, and for CO and N_2 those of Winkler.

of hyposulfite which usually adheres to it at the top of the chamber. The hyposulfite solution is drawn down into the trap bulb, and the volume of the residual N_2 is read, as shown in figure 31. To find the oxygen volume this N_2 volume is subtracted from the total volume of $O_2 + N_2$ observed on the first reading.

Calculation. The oxygen content is calculated by multiplying the observed oxygen volume by the factor indicated in table 26. The factors there for O_2 are calculated by the equation given on page 249, but with the proper values for α'_{O_2} (from table 27) in place of α'_{CO_2}, and without the reabsorption factor i, since reabsorption of the relatively insoluble O_2 is negligible if the readings are made without unnecessary delay.

Remarks. Duplicates by this method may be expected to agree within ± 0.25 volume per cent of oxygen, corresponding to a variation of ± 0.005 cc. in the reading. When a greater accuracy is necessary results with about one-fifth this variability may be obtained with the manometric apparatus.

When rapidity of analysis is desirable some time may be saved, with some loss of accuracy, by omitting the absorption of oxygen with hyposulfite and measuring only the total $O_2 + N_2$ volume. This is multiplied by the proper factor from table 26, and from the result is deducted a correction of 1.2 volume percent, which represents the N_2 content found in circulating blood analyzed by this procedure.

With the volumetric apparatus used for oxygen determinations the Shohl trap (fig. 28) is highly desirable in order to prevent high results from entrance of traces of air through the rubber tube when the apparatus is evacuated. Such error is 4 times as great when the O_2 is not absorbed, since the nitrogen content of the air is 4-fold the oxygen.

In the above oxygen determinations it might seem that one step in the manipulation, the absorption of carbon dioxide prior to the oxygen measurement, might be omitted by makng the ferricyande solution sufficiently alkaline to prevent escape of carbon dioxide into the gas phase. In fact ammonia was added for this purpose by Barcroft and Haldane (4), and in the first paper by Van Slyke (45). Van Slyke and Stadie (64) found, however, that an alkalinity sufficient to prevent evolution of carbon dioxide with certainty may interfere with the complete extraction of the oxygen, or accelerate consumption of oxygen by some reducing agent present, and cause low results.

CARBON MONOXIDE, OR CARBON MONOXIDE AND OXYGEN, IN BLOOD. VAN SLYKE AND SALVESEN (61); O'BRIEN AND PARKER (25)

Reagents

Acid saponin-ferricyanide solution.
1 N NaOH solution, gas free.
20 per cent hyposulfite solution, gas-free.
Caprylic alcohol.
These are all described under "General reagents."

Procedure

The determination up to the point where the extraction of the gases is finished, is performed exactly like the determination of oxygen content, except that the "acid saponin-ferricyanide solution" (p. 234) is used to free the oxygen and carbon monoxide. The affinity of carbon monoxide for hemoglobin is so great that the neutral ferricyanide solution will not free it all within a reasonable time. By making the reagent solution acid, however, and increasing the ferricyanide content, conditions are obtained which permit complete extraction of the carbon monoxide in three minutes.

After the extraction is complete the carbon dioxide is absorbed with sodium hydroxide solution, as in the oxygen determination, and the blood-reagent mixture is drawn down into the bulb below the lower cock. The mercury is then let back into the chamber until the pressure is but slightly negative, (leveling bulb in position *2* of figure 26) and the oxygen is absorbed with hyposulfite, as described above. If the blood were present together with any considerable amount of carbon monoxide gas during the hyposulfite treatment some reabsorption of the extracted carbon monoxide would occur. The hyposulfite reduces all the methemoglobin formed by the ferricyanide action to reduced hemoglobin which can recombine with carbon monoxide, for which it has a tremendous affinity. In order to prevent such recombination, therefore, the hemoglobin solution is drawn out of the chamber before the hyposulfite is let in. The oxygen can be measured, as described above, by noting the volume of gas absorbed by the hyposulfite.

After the oxygen has been absorbed by the hyposulfite solution, the latter is drawn down into the lower bulb with the other solutions, as it would otherwise precipitate the cuprous chloride solution next added. The volume of the $CO + N_2$ left in the chamber is measured.

With the leveling bulb a little higher than position 2 (fig. 26), and the gas under slightly negative pressure, 5 cc. of the cuprous chloride solution are run slowly into the chamber in the manner described above for admission of hyposulfite. The admission of the cuprous chloride solution should take two minutes or longer, in order to allow time for complete absorption of the carbon monoxide. The volume of N_2 left in the chamber is then measured ***without removing the cuprous chloride solution.*** There are two reasons for leaving the cuprous chloride solution in the chamber at this time. First, if it were mixed with the other reagents in the lower bulb it would form a heavy precipitate with

them which would be hard to remove. Second, the combination of carbon monoxide with cuprous chloride is a loose reversible one, and if the chamber were subjected to the high vacuum necessary to draw the solution into the lower bulb a considerable part of the carbon monoxide would bubble out of the cuprous chloride solution and return to the gas phase. Consequently the residual nitrogen gas volume is read over the cuprous chloride solution, the level of the mercury in the leveling bulb being held above the mercury meniscus in the chamber, by 1/11th the height of the cuprous chloride solution column, as shown in figure 31. In the present case one estimates 1/11th instead of 1/13th the height of the solution column because the specific gravity of the solution, 1.22, is 1/11th that of mercury.

The carbon monoxide volume is the difference of the gas volumes observed before and after absorption with the cuprous chloride solution. From it the carbon monoxide content of the blood is calculated by the factor in table 26.

When small amounts of carbon monoxide in blood are to be determined with accuracy it is desirable to take 5 cc. of blood and 2.5 times the amount of each reagent solution recommended in the above description, except the CuCl solution. The factors in table 26 are multiplied by 0.4 when a blood sample of 5 instead of 2 cc. is used.

HEMOGLOBIN BY OXYGEN CAPACITY, WITH AERATION OF BLOOD IN THE CHAMBER OF THE VOLUMETRIC APPARATUS. LUNDSGAARD AND MØLLER (21)

The ferricyanide reagent is first deaerated and stored in the small bulb at the bottom of the chamber. The blood is then saturated with air in the chamber, the undissolved air is ejected, and the O_2 content of the blood is determined. Since saturation with atmospheric air causes 100 per cent of the hemoglobin to become HbO_2, the O_2 content of the blood, when corrected for a slight amount of physically dissolved O_2, serves as a direct measure of the hemoglobin.

Reagents

Saponin solution, 0.3 per cent.

Potassium ferricyanide solution, saturated.

Sodium hyposulfite solution and *N sodium hydroxide*, both air-free, as described under "General reagents."

Caprylic alcohol.

Procedure

Five cubic centimeters of the saponin solution and 2 or 3 drops of caprylic alcohol in the chamber of the volumetric apparatus are freed of air as above described for determination of oxygen. The air-free solution is then drawn into the bulb below the extraction chamber and kept there by closing the lower cock. Through tube *C* of figure 26 mercury is admitted and is permitted to rise slowly in the chamber, collecting a slight film of solution from the walls. The solution thus collected on the mercury surface is ejected through the capillary outlet of the upper cock and the cup is washed with water. A 2 cc. sample of blood and a drop of octyl alcohol are introduced into the chamber. ***With the upper cock left open*** to connect the chamber with the air in the cup, the mercury is withdrawn from the chamber through tube *c* (fig. 26). The lower cock is closed and the chamber is shaken for three minutes to saturate the blood with the air. It is necessary to oxygenate thus the ***undiluted*** blood: if the blood were laked with the saponin solution before aeration, the oxygenation would be only about 95 per cent complete.

After the three-minute aeration is finished mercury is readmitted through tube *c* until all the air has been ejected from the chamber. Just enough blood is run up out of the chamber to fill the capillary at the bottom of the cup. Then 0.1 cc. of a solution containing 20 grams of potassium ferricyanide per 100 cc. is placed in the bottom of the cup and is run down into the blood, leaving enough above the cock to fill the capillary. A drop of mercury is then run into the bore of the stopper to seal it, and all mercury is withdrawn from the chamber through tube *c* (fig. 26). The saponin solution that has been held in the trap bulb below the lower cock is now admitted to the chamber. The oxygen is extracted from the blood solution in the usual manner and measured after absorption of the carbon dioxide. One may either measure the $O_2 + N_2$ together and correct for the N_2, or more exactly, one may measure the O_2 by absorption with hyposulfite solution, as described above for oxygen determination.

Calculation. The O_2 or $O_2 + N_2$ content of the blood is calculated by the proper factor from table 26. From the volume per cent content of total O_2 subtract 0.5 volume per cent for physically dissolved O_2 to obtain oxygen combined as HbO_2. Or from the total $O_2 + N_2$ content subtract 1.9 volume per cent for the O_2 and N_2 in physical solution. The corrections for N_2 and physically dissolved O_2 have been computed as described by Van Slyke and Neill (59).

OXYGEN CAPACITY DETERMINATION WITH PRELIMINARY OXYGENATION OF THE BLOOD IN A SEPARATE VESSEL

Instead of saturating the blood with air in the volumetric apparatus itself, it may be first saturated in a separate vessel, from which samples are drawn for analysis. A convenient vessel for this purpose is a cylindrical separatory funnel or centrifuge tube of a volume 25 to 50 times that of the blood sample to be aerated. The saturation is performed by rotating the cylinder in a horizontal position for a few minutes, so that the blood spreads over the surface in a thin film. A convenient arrangement for rotating the saturating vessels by motor was devised by Stadie (38), and is shown in figure 34. The oxygen content of a sample of the saturated blood is determined as described above on page 257.

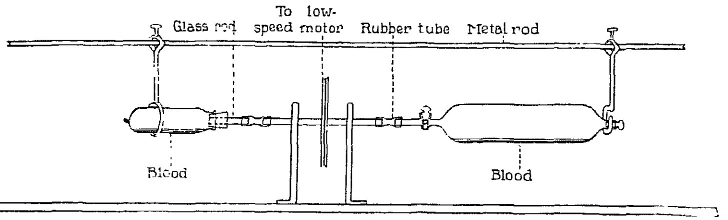


FIG. 34. Stadie's mechanical rotator for saturating blood with air. From Stadie (38)

The calculation is the same as in the Lundsgaard-Møller method described above.

Aeration in a separate vessel permits one to oxygenate at once enough blood for duplicate analyses, and to have the gas apparatus during the aeration free for other analyses. Consequently, when the supply of blood is adequate and a Stadie rotator is available, aeration in a separate vessel is more convenient than aeration in the chamber of the gas apparatus.

DETERMINATION OF HEMOGLOBIN BY CARBON MONOXIDE CAPACITY METHOD

The method of Van Slyke and Hiller can be applied as described on pages 341–347 for the manometric apparatus with 2-cc. blood samples. The only changes necessary are that the gas readings are made in the volumetric apparatus, as described on page 245, and that the calculations are by the factors in table 26. When the carbon monoxide capacity method is used no corrections are necessary for dissolved O_2 or $O_2 + N_2$, since these gases are completely removed in the preliminary extraction.

USE OF VOLUMETRIC APPARATUS IN ANALYSES DESCRIBED FOR MANOMETRIC APPARATUS

Any of the analyses described below for the manometric apparatus can be performed also with the volumetric apparatus. The volumetric apparatus does not permit the same fine degree of precision, but when provided with a Shohl air trap it is capable of yielding good reproducible results with any of these analyses except those with the smallest amounts of gas to measure.

The tables of calculation factors given for use with the 50 cc. manometric apparatus can be applied to the 50 cc. volumetric apparatus by the following formula.

$$\text{Milligrams of substance in sample, or per 100 cc. blood, etc.} = V \times \frac{F}{a} \times (B - W)$$

V is the volume, in cubic centimeters, of gas in the volumetric apparatus, observed at barometric pressure, and corrected by subtracting the volume of gas, if any, obtained in blank analysis of the reagents.

B is the barometric pressure in millimeters of mercury, *uncorrected* for temperature effect on mercury expansion. This correction has been included in the calculation of F.

W is the vapor tension of water (see table 18, p. 163).

F is the manometric factor for the same temperature and analysis, by which the gas pressure in the manometric apparatus is multiplied to obtain milligrams of substance in sample, or per 100 cc. of blood, etc. The values of F are given in the tables with the manometric analyses.

a is the volume, either 2.0 or 0.5 cc., in manometric apparatus at which the pressures are read in the manometric analysis.

If any number of analyses of a given type are to be done with the volumetric in place of the manometric apparatus, it is worth while to make a table of values of $\frac{F}{a}$ for that analysis covering the temperature range of the laboratory. Such a table is made simply by dividing each factor in the manometric table by the value of a, either 0.5 or 2.0.

The fact that in place of calculating "amount of substance determined" as $P \times F$ from manometric pressure P and factor F, one can calculate the substance as $\frac{V\,F\,(B - W)}{a}$ can be shown as follows. If a given amount of gas is measured at the same temperature both in the manometric apparatus, at volume a and pressure P, and in the volumetric apparatus, at volume V and pressure $B - W$, we shall have by the gas laws:

$$V\ (B - W) = a\ P$$

whence

$$P = V\ (B - W)$$

and

$$\text{Amount of substance determined} = P\ F = \frac{V\ (B - W)}{a} \times F = V\ \frac{F}{a}\ (B - W).$$

The Manometric Apparatus and the Technique of Its Use (48, 59)[4]

The manometric apparatus is required when a higher degree of accuracy than that of the volumetric apparatus is necessary. This is the case when accurate determination of both carbon dioxide and oxygen in a single 1 cc. sample of blood is desired, or micro-determination in 0.2-cc. samples, or when for any reason small variations are significant. The use of the manometric apparatus also simplifies the calculations, since the barometric pressure as a factor is eliminated.

The manometric method resembles the volumetric one, except for the manner in which the gases are measured. In the volumetric method the pressure is brought always to atmospheric and the volume of gas is read on a scale, while in the manometric method the gas in brought to an arbitrarily chosen volume and the amount of the gas is determined from the pressure exerted on a manometer. When the mass of gas is estimated from its volume at atmospheric pressure, as is done with the volumetric apparatus, the error in small volume readings may be 10 or even 100 times the error of the pressure measurement (on the barometer). The advantage of the manometric method is that it permits one to choose the magnitude of both the volume and the pressure which are measured. One may accordingly fix them within such limits that the errors in measuring both are of the same order of magnitude. Thereby the percentage error in gas measurement becomes the minimum attainable with given absolute errors in pressure and volume. In consequence the usual deviations between duplicate results of

[4] This apparatus can not be introduced without acknowledging its debt to the skill and precision of John Plazin, the writer's technical assistant for the past seventeen years. Plazin constructed the first manometric apparatus, and performed many of the analyses in the development of manometric methods for the blood gases, and for other substances, especially, urea, amino nitrogen, and the micro Kjeldahl method for total nitrogen.—D. D. V. S., 1931.

ordinary blood gas analyses is, with the same size of blood samples, about 1/5 as great with the manometric as with the volumetric apparatus.

In the manometric apparatus the extracted gases are for measurement reduced to a volume, a, of 0.5 or 2.0 cc. (fig. 35) by admission of mercury from the leveling bulb, and a reading p_1 is made on the manometer. The gas to be determined is then removed or absorbed, and the reading p_2 is taken, with the unabsorbed gas at the same a cubic centimeter volume. The pressure fall $p_1 - p_2$ millimeters of mercury, between the two readings therefore is the pressure which the determined gas exerted at a cubic centimeter volume. The volume which the gas would occupy at 0°, 760 mm. is calculated by mulitplying this pressure fall by a single factor, which is a function of the temperature. No corrections are required for vapor tension or for capillary attraction in the manometer tube, since these factors are the same at both readings. It is assumed that the temperature in the apparatus is the same when the p_1 and p_2 readings are taken. The time interval between the two readings is so brief (one to four minutes) that sufficient constancy is attained (within 0.1°C.) by the insulation afforded by the waterjacket.

The ease and accuracy with which the manometric apparatus makes possible the measurement of small mounts of gas evolved from solutions has led to extension of its use to micro determinations of substances other than the blood gases. The apparatus can be used in any determination in which the final product is a gas suitable for measurement, or will enter into a quantitative reaction producing such a gas. Thereby the measurement is based, as in gravimetric analysis, on direct observation of the amount of substance obtained, independent of comparison with standard solutions by titration, colorimetry, or otherwise. This advantage of directness and freedom from dependence upon the accuracy of standards is combined with the rapidity and ease of a final measurement by volume. It is probable that the number of substances which can be made to enter reactions giving quantitative yields of gas approaches the number which give quantitative yields of precipitate for weighing. For example, since iodine (3*c*) and iodate (58) can be determined from the amount of nitrogen gas that they evolve by oxidation of hydrazine in the apparatus, the latter can be used for microanalysis in any determination based on iodimetry. Technique with the manometric apparatus has been developed for micro determinations of organic nitrogen and carbon, urea, amino nitrogen, iodates, sulfates, total base, sugar, calcium, lactic acid, nitrites, and potassium, and for analyses of gas mixtures.

Results of high accuracy can be obtained in these determinations by any

analyst who has learned to handle the apparatus. To become acquainted with the details of manipulation it is desirable to spend a few hours at the start under the tuition of one who is already initiated. The different individual analyses can then be acquired without difficulty by following the published procedures. If there is no opportunity to learn the apparatus from a fellow analyst, one can, with a little more time, acquire the technique by following the directions given below.

The only disadvantage of the manometric apparatus compared with the volumetric lies in the greater initial cost of the former. The metallic wheel and shaft for shaking the chamber require the service of a good mechanic. On the other hand the simplicity of the extraction chamber in the manometric apparatus makes it possible for one with ordinary skill in sealing glass tubes to construct the glass parts of this apparatus, including the manometer, from a 50-cc. pipette, 4 glass cocks and some tubing.

DETAILS OF MANOMETRIC APPARATUS

The details of the apparatus are apparent from figures 35, 36, and 37. Accessory apparatus which is desirable has been listed on page 223.

Rubber tubing. The rubber tube which connects the leveling bulb should be of heavy walled "nitrometer" tubing. It needs to be changed about once a year. One should be able to use it for a full day of constant work before enough air to require expulsion leaks through the rubber and accumulates in the trap tube above the + joint. If the rubber is used too long it becomes porous.

The *extraction chamber* is simpler than that of the volumetric apparatus; it is calibrated only at three points, usually 0.5, 2.0 and 50.0 cc., and has no cock at the bottom. The *mercury seal*, shown in figure 35 around the junction of the two glass tubes at the bottom of the chamber, is necessary. Even thick pressure tubing, exposed to the air, permits diffusion, during the extraction *in vacuo*, of sufficient amounts of air to affect pressure readings as accurate as those that can be made with this apparatus. The mercury seal makes unnecessary the Shohl trap recommended above for the volumetric apparatus. The mercury forming the seal must cover, not only the sides, but also the ends of the short, thick, rubber tube which connects the glass tubes.

The volume and shape of the chamber are matters of convenience. For all the analyses described in this chapter convenient magnitudes are 50 cc. for the total volume and 2.0 and 0.5 cc. for the volumes at which the gas pressures are measured (see fig. 35). At 20°, 0.01 cc. of gas at 2-cc. volume gives a reading of 3.9 mm. pressure. For very small amounts of gas,

measurement of the pressure at 0.5 cc. volume is desirable. At this volume 0.01 cc. of gas exerts about 16 mm. pressure.

After use for a year or two the *cock at the top of the chamber* may begin to need unduly frequent greasing to prevent leakage. It can usually be repaired by grinding with fine emery powder. This cock should serve for a

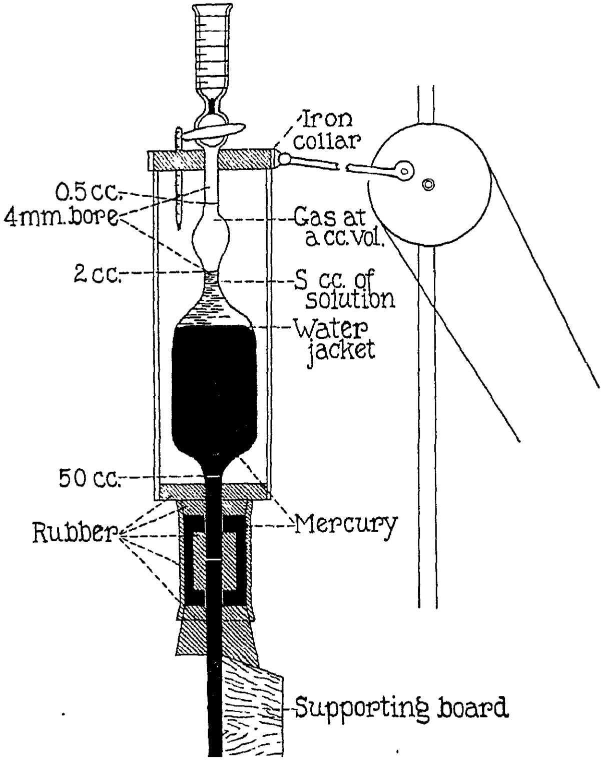


FIG. 35. Extraction chamber of manometric apparatus, showing mercury sealed rubber joint at bottom and attachment of Stadie shaker at top. Gas is shown at 2-cc. volume for pressure reading. The glass stem at the bottom of the chamber must be visible for about 10 mm. below the 50-cc. mark. From Van Slyke and Neill (59).

dozen or more analyses without renewing its grease. If it requires greasing oftener it should be returned to the maker.

The bore of the cock leading to the cup above the chamber should be 1.2 to 1.3 millimeters in diameter. The other bore, through the curved capillary outlet (see figure 52, page 344) should be 1.5–1.6 mm.

The *manometer scale* may be a meter stick or metallic tape attached to the board behind the tube, but the scale is preferably etched on the tube itself. The marks are 1 mm. apart, and are in the form of semi-circles pass-

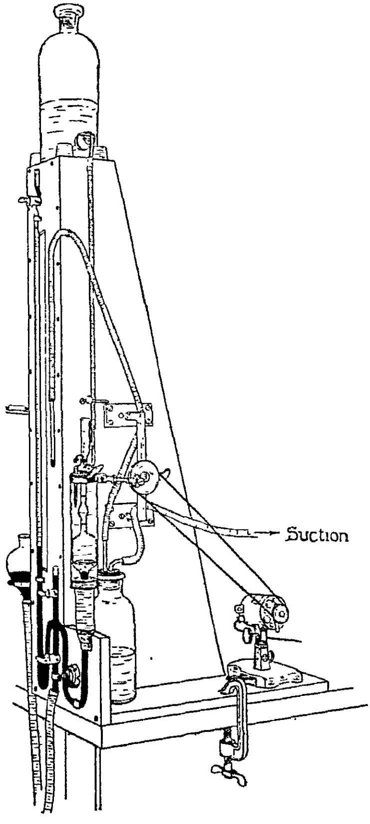


FIG. 36. Manometric apparatus clamped to table. The bottle at the top holds distilled water. The lower bottle is to receive waste solutions drawn out of the chamber after analyses. The most convenient way to transfer solutions to waste bottle is to force them into the cup above the chamber and draw them over into the bottle by suction. From Van Slyke (48).

ing around the tube from the middle of the front to the back. The centimeter and half centimeter marks are complete rings. A white background is placed behind such a manometer tube, or it may be illuminated from the rear by removing a strip of the supporting board and inserting a plate of

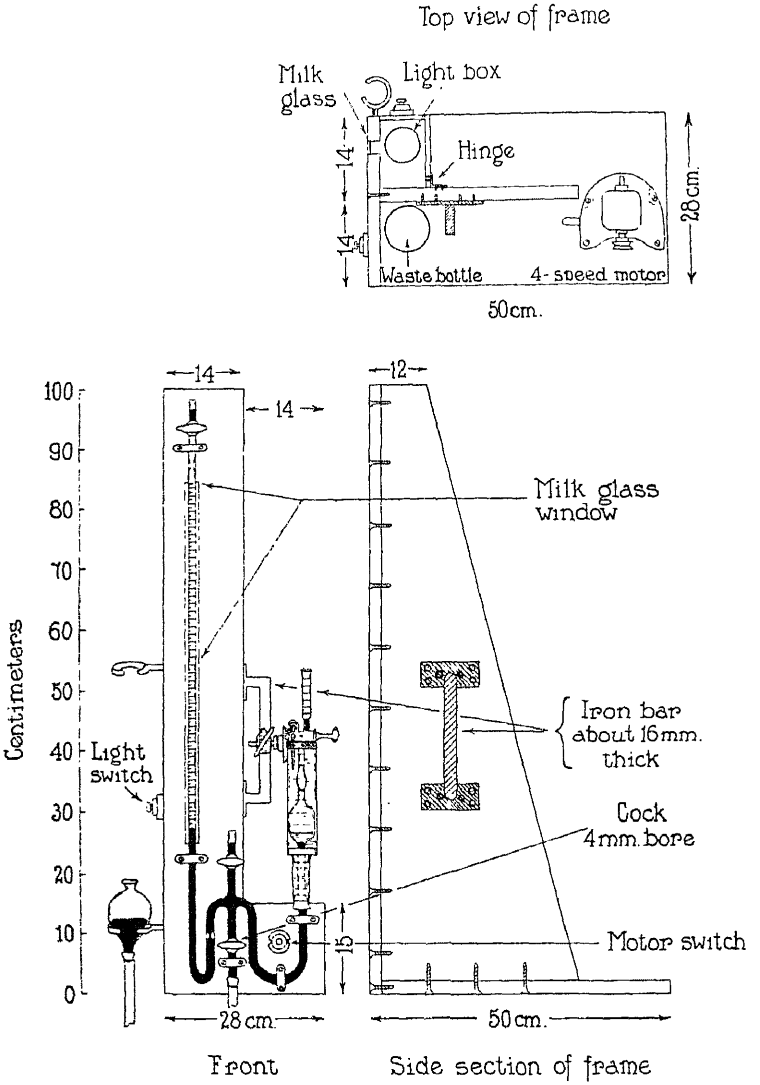


FIG. 37. Diagram of portable manometric apparatus. The light box contains a long cylindrical electric bulb which illuminates the manometer. The switch on the side of the wooden frame turns on the light: the switch in front turns on the motor. The zero point on the manometer tube should be at a level a few millimeters below the 50-cc. mark on the extraction chamber. From Van Slyke (48).

frosted glass behind which are electric lights. The zero point on the scale should be slightly *below the 50-cc. mark* on the extraction chamber. The length of the scale should be about 650 mm.

The cock above the + joint (fig. 37) serves from time to time to let out air, which diffuses through the rubber tube from the leveling bulb. This air gradually accumulates under the cock, where the space in the ascending limb of the + joint serves as a trap.

The manometer tube (in front of milk glass window in figure 37) is of 4 to 5 mm. inner diameter. It is closed with a cock at the top. The air is expelled before the manometer is used, so that there is no atmospheric pressure on the mercury surface in the tube. The tube is contracted to 1-mm. bore a little below the cock at the top, as shown in figure 37, to lessen the force with which the rising mercury strikes the cock. The inner bore below, between manometer and + joint, is again contracted for a short distance to about 1.5 mm., as shown in figure 37, to minimize oscillation of the mercury when readings are taken.

Slight amounts of moisture find their way into the manometer as the mercury flows back and forth from the analysis chamber. Some error from variation of the vapor pressure of this moisture might occur if it were left in the manometer tube, as the latter is not protected against sudden temperature changes from the room air. To absorb the water vapor a few drops of a *water absorbing fluid*, such as trimethylene or dimethylene glycol (glycerine derivatives sold to prevent the freezing of water in automobile radiators) are admitted through the cock at the top and permitted to flow down the tube for about 10 cm. Mercury is then forced up through the cock, leaving behind enough of the dehydrating fluid to wet the upper end of the manometer, but not enough to flow down and interfere with readings of the mercury meniscus. The dehydrating fluid is renewed occasionally in this manner. Concentrated sulfuric acid can be used, but is undesirable because it gradually chars the grease on the cock.

One may test the apparatus as follows to find whether the drying fluid is functioning properly. With the extraction chamber closed at the top and empty of gases and of fluid except the moisture that is always on its walls, one lowers the mercury surface in the chamber to the 2-cc. mark. The 4-mm. cock leading to the mercury leveling bulb (fig. 37) is now closed, and the level of the mercury in the chamber is compared with that in the manometer. If the drying fluid in the manometer is functioning, the mercury surface in the manometer will be above that in the chamber by a height nearly equal to the vapor

pressure of water, 15 to 25 mm. depending on the room temperature (see table 18 on p. 163). If this is not the case, one should renew the drying fluid as directed above. It is convenient to put a permanent mark on the manometer at the point level with the 2-cc. mark in the chamber. One can then make the test in a few seconds by lowering the mercury in the otherwise empty chamber to the 2-cc. mark, and noting whether the mercury in the manometer is at a proper level above the mark. It is well to make this test before starting each day's analyses.

One must never lower the leveling bulb below the position it has in figure 37 when the cock at the top of the extraction chamber is open to the air. If one does this the mercury in the chamber will all flow into the leveling bulb, and air and whatever solution is in the chamber will be drawn over into the manometer. This accident occurs once to every one who uses the apparatus. When it happens one must clean out the manometer.

To clean the manometer open the cock at the top, draw the mercury as completely as possible into the leveling bulb, and disconnect the rubber tube leading to the bulb. Then attach a tube from a suction flask to the place below the + joint (fig. 37) where the tube of the leveling bulb was attached. Apply strong suction, and draw through the manometer from the opened top first water, then, if the manometer is soiled with dirt or grease, alcoholic KOH solution, then acetone. Finally draw air through until the acetone is removed. Instead of alcoholic KOH one may clean the tubes with chromic-sulfuric acid, followed by water and acetone.

When the manometer is refilled with mercury, the tube is evacuated several times by lowering the leveling bulb in order to detach the slight film of air adherent to the glass. The air bubble thus obtained is expelled through the cock at the top. The drying fluid is then put in as above directed.

The *shaking arrangement* is a modification of that of Stadie (38) illustrated above (fig. 32) in connection with the volumetric apparatus. It is highly desirable to have a smoothly running and noiseless shaking device. For this purpose it is essential to use ball bearings for both bearings in the driving wheel. Shaking arrangements so equipped are provided by manufacturers of the apparatus. The driving wheel should be run at a speed of *300 to 400 revolutions per minute,* in order to complete gas extractions in the periods stated in the directions for various analyses given below.

Alternative open manometer

In the original form of apparatus first used by Van Slyke and Neill (59) there was no cock at the top of the manometer. The manometer tube was open to the air, as shown in figure 38. The bulb at the top served to prevent loss of mercury by spurting when the leveling bulb was raised above the reaction chamber. A scale a meter long was used, the

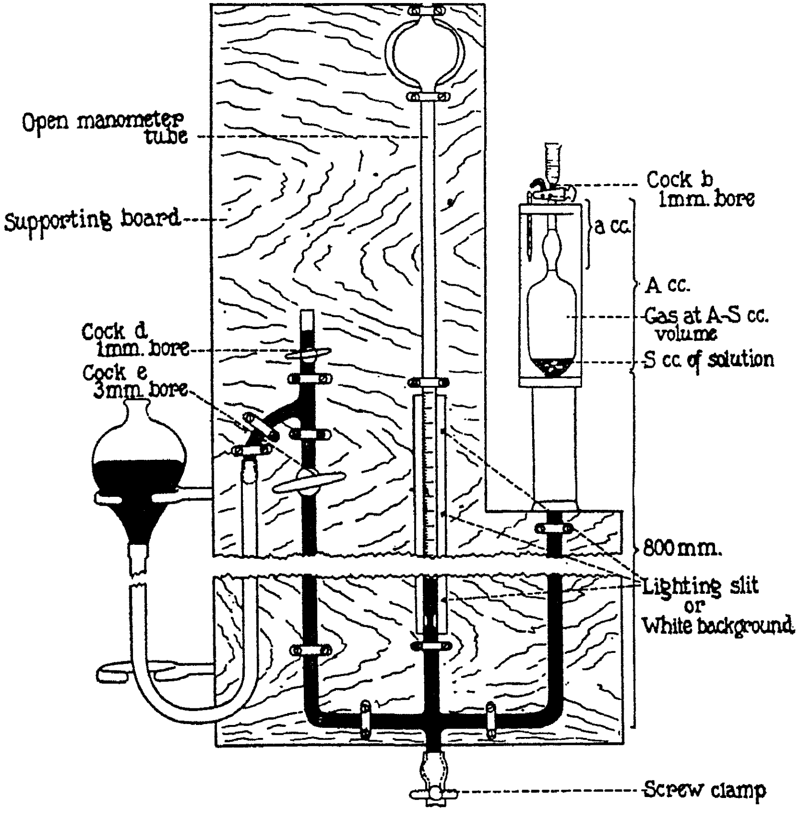


FIG. 38. Manometric apparatus with open manometer. Solution, in bottom of evacuated chamber, is ready for extraction of gases. From Van Slyke and Neill (59).

zero point being about 800 mm. below the 50-cc. mark on the reaction chamber. Zero readings made when the chamber is free of gases are, with such an apparatus, about 760 mm. below the 2 or 0.5-cc. mark on the chamber, and consequently are taken at a point on the manometer scale about level with the analyst's knee. Because of the inconvenience, in a long series of analyses, of making numerous readings at such a low level, the cock at the top of the manometer was added. This removed one atmosphere of pressure from the

mercury in the manometer, and consequently raised all the readings 760 mm. farther above the floor.[5] Another advantage was to shorten the apparatus so that it was all above the level of the table, and, being much more compact, could be made in a portable form readily transferred from one desk to another. These advantages were so marked for anyone using the apparatus in routine work that the long open manometer has been practically abandoned for the closed type described in the preceding pages. For some conditions, however, it may be desirable to revert to the open type. The relative conveniences of the two may be compared as follows.

The open manometer has the following advantages: (1) There is no possibility of drawing water from the chamber over into the manometer tube. Such an occurrence is prevented by the great length of tubing below the level of the chamber. (2) A certain, if small, amount of attention required to see that the closed manometer is free of air and moisture (use of dehydrating fluid) is not required with the open manometer. (3) In case the open manometer becomes dirty it can be cleaned in a moment by means of an iron or, better, steel wire ramrod bearing a piece of cotton at its end. The closed manometer must be emptied and cleaned with alcoholic potash or chromic acid mixture. (4) The apparatus with open manometer is more readily emptied of mercury because it has an outlet at the lowest point of the system of tubing.

The closed manometer wins on the following points: (1) The zero readings are made at a convenient level above the desk top, instead of at the level of the knee. (2) The closed manometer permits making the entire apparatus in a compact, portable unit. (3) Because of the smaller weight of mercury in the shorter tubes of the closed manometer, it is slightly easier to avoid oscillation of the mercury while adjusting gas volume in the chamber before readings. (4) The zero point of the closed manometer does not change with barometric pressure. This is of some importance when one is running a series of sugar, urea, or other determinations of the type in which one preliminary blank analysis is used to fix the zero point for an entire series of analyses.

For an analyst who uses the apparatus much, the conveniences of the closed manometer outweigh the, in actual practice, relatively unimportant advantages of the open manometer. The open manometer, however, requires less attention to keep it in order, and is practically accident-proof. It may be the more desirable type to put into the hands of students.

DETAILS OF GENERAL TECHNIQUE FOR MANOMETRIC APPARATUS

A number of points have already been discussed on page 236 under "technique common to both volumetric and manometric apparatus." In addition the following apply to the manometric.

Lubrication of cocks (see also pages 47 and 238)

When the 4-mm. cock between the leveling bulb and the gas chamber is to be lubricated, the leveling bulb is hung at a level a little below

[5] The closed manometer really owes its origin to our laboratory comrade of many years, Glenn E. Cullen. The numerous genuflexions required during a day's work in reading the low lying zero points of the open tube told heavily on Cullen's jovial proportions, and the laboratory felt so much the loss of his usual contagious spirits, that the more humane closed tube had to be devised.

this cock. The small cock above the + joint between the manometer and chamber is then opened. The mercury levels in the tubes then adjust themselves so that the 4-mm. cock can be removed without spilling any of the metal.

Incomplete lubrication should never be tolerated for a single analysis in either of the cocks. Ease and precision in the analysis depend upon having them both in smooth and perfect working order. They must not only be proof against leakage, they must also turn smoothly, without the slightest tendency to stick, or precise work will be difficult. As soon as any lack of ease or smoothness is noted, the cock involved should be greased before another analysis is started. The cock at the top of the chamber should, if well made, serve for at least a dozen analyses before lubrication needs to be repeated, and the 4 mm. mercury cock for many more. Occasionally a defective cock is encountered which needs greasing oftener.

Adjustment of gas volume

The precision of the method rests on exactness in bringing the gas to 0.5 or 2.0-cc. volume (figure 35) before each pressure is measured. The calibration rings at this points should be colored to make them readily visible through the water jacket. It is an advantage to locate the apparatus so that light falls on it from behind the observer. A white background for the reading can be secured by attaching a strip of adhesive tape 7 or 8 cm. wide to the back of the water jacket, or by locating the apparatus with a white wall behind it. Light reflected back from the white surface across the meniscus facilitates a sharp reading. By far the best lighting arrangement, almost indispensable for the most accurate results with the deep red solutions of whole blood, is a "frosted" light placed about 50 cm. behind the chamber. The use of a lens (an ordinary reading glass serves well) to observe the meniscus as it comes to the 2 or 0.5-cc. mark of the chamber is a decided aid to precision, especially in micro analysis. See also "Reading meniscus of solution in chamber," pages 243–4.

In CO_2 analyses. In reducing the gas volume after extraction, the analyst must avoid jerky starting or stopping of the mercury inflow, because either will cause the mercury in the chamber to oscillate up and down, with resultant increase in the amount of CO_2 that is reabsorbed by the solution as it rises in the chamber. The cock to admit mercury is opened with a ***smooth gradual motion,*** and the mercury is allowed to rise at a fairly rapid rate through the middle three-fourths of the chamber. Then ***gradually*** the cock is closed to reduce the rate of flow,

making the latter so slow by the time the 0.5 or 2-cc. mark is reached that there is no difficulty in stopping exactly at the mark. The entire operation takes about forty seconds. If it is unduly prolonged reabsorption of carbon dioxide is increased. If the fluid meniscus is not stopped exactly at the mark, readjustment must not be attempted, as moving the fluid up and down in the chamber will result in reabsorption of more CO_2 than is allowed for by the *i* correction discussed below. It is necessary to lower the mercury again to the 50-cc. mark, extract again for a minute, and raise the meniscus again to the 0.5 or 2-cc. mark.

However, after a little experience has been gained such repetition is practically never necessary.

In bringing the gas to 2.0 or 0.5-cc. volume when only gases with relatively low solubilities, such as O_2, N_2, H_2, and CO are present, reabsorption is so nearly negligible that, if one does not succeed in stopping exactly at the mark, readjustment may be carried out without repeating the extraction.

Temperature adjustment

It is necessary for accuracy that no significant temperature change occur in the chamber of the apparatus during an analysis. If before analyses are begun the temperature indicated by the thermometer in the water jacket of the chamber is different by more than 1° from that of a thermometer in the air of the room at the same height from the floor, the water jacket should be cooled or warmed to eliminate the difference. This is accomplished by wrapping the jacket for a minute or more in a towel soaked with hot or cold water. The towel is removed, the chamber is shaken, and the thermometer in the water jacket is read to see whether the desired temperature is reached.

Doors, drafts, etc. in the room are arranged so that the room temperature is as constant as possible.

Manometer readings

The manometer is usually read to 0.1 mm. A reading glass is of assistance in estimating the tenths of a millimeter division. One must be careful to read with the eye on an exact level with the mercury meniscus in the manometer in order to avoid error due to parallax. Such error may be excluded by the use of a millimeter scale of rings etched on the manometer tube, or by placing a strip of mirror glass behind it.

Ejection of gas without loss of solution

In some analyses, either at the beginning, as in the micro Kjeldahl, or before the final manometer reading at the end, it is necessary to eject quantitively the gas in the chamber, without either losing any of the solution or exposing it to air. The most satisfactory technique is the following:

Bring the pressure in the chamber to approximately atmospheric by connecting the chamber with the leveling bulb and holding the latter at a height only enough above the mercury surface in the chamber to balance the column of water in the latter (see "Reading gas volumes at atmospheric pressure in volumetric apparatus," p. 244). The mercury cock connecting with the leveling bulb is then closed and the cock at the top of the chamber is opened wide. The contents of the chamber remain quiet, as the pressure was already atmospheric. Now with the leveling bulb resting in the ring above the chamber level, the mercury cock connecting bulb and chamber is slowly opened. The fluid in the chamber rises slowly. By closing the mercury cock the rise is stopped when solution from the chamber has just filled the capillary leading from the top of the chamber to the cup above.

Determination of c correction by blank analysis

From the $p_1 - p_2$ value observed in an analysis (see p. 268) it is usually necessary, for precise results, to deduct a correction which in the calculation formulae used in this chapter is indicated by c.

The c correction is obtained by performing a blank analysis, in a manner directed for each type of determination. The $p_1 - p_2$ value found in the blank constitutes the c correction.

The c correction may be the sum of two components. One of them is a slight amount of gas which may be yielded by the reagents themselves.

The other component is the fall in manometer reading caused, even when no gas is removed between the p_1 and p_2 readings, by the introduction of a given volume of absorbent solution, such as the 0.5 or 1.0 cc. of 1 N sodium hydroxide used to absorb CO_2 in blood gas determinations. The introduction of solution between the two manometer readings p_1 and p_2 lowers the p_2 value merely by increasing the volume of fluid between the mercury surface and the water meniscus at the moment of reading. Thereby the level of the mercury surface in the chamber, and hence also in the manometer, is lowered. The extent of this effect can be determined by means of blank analyses, in which, with the same amount of reagents but no gas in the chamber, the usual amount of absorbent solution is admitted. The manom-

eter is read, with the water meniscus in the chamber at the same mark before and after the admission of the absorbent solution. The difference between the two readings measures this component of the c correction. The shape of the apparatus causes the area of the meniscus of the mercury in the chamber to vary according to the volume of solution present, and according to the location of the water meniscus (at the 0.5 or 2.0-cc. mark), such variations causing the mercury meniscus to be located at points of different cross section in the conical upper portion of the chamber. The fall in p_2 caused by 1 cc. of added solution in the 50-cc. apparatus may accordingly be from 1 to 4 mm., depending on the location of the water meniscus, the shape of the chamber, and the volume of solution in it.

When the final manometer reading is obtained after expulsion of the gases instead of after addition of an absorbing solution, there is of course no correction of this sort, although there may be a correction for gas from the reagents. Both corrections are automatically included in the c correction determined in blank analyses.[6]

Use of the modified Hempel pipette

In some analyses, viz., the determination of carbon monoxide by the Sendroy-Liu method and the determination of amino nitrogen, it is necessary to withdraw a gas mixture from the manometric chamber in order to absorb one or more gases, and then return the unabsorbed gases to the chamber for measurement. For this purpose a modified Hempel pipette has been devised (figs. 11, 48, 55, 56, 57).

One's ability to transfer gas to and from the modified Hempel pipette easily and without the loss of 0.001 cc. of gas depends upon proper construction of the latter. The rubber tip, made from soft tubing of 5-mm. outer diameter and 1-mm. bore, should project a hair's breadth below the glass tip of the pipette, and should be so shaped that it fits snugly into the bottom of the cup of the manometric chamber. The bore of the capillaries of the pipette should be between 1.0 and 1.2 mm. In particular the three-way cock must be accurately ground, and the bore through the stopper must main-

[6] Van Slyke and Neill in their original paper defined as the c correction only that component caused by the fall of the mercury surface in the chamber when absorbent solution is added. Later, when analyses with corrections also for gas evolved by reagents were devised, "c" was used to express the total correction. This extension of its meaning may have caused some confusion. In the calculation formulae for manometric analyses in this chapter c is used throughout to express the total correction, sometimes due solely to gases from the reagents, sometimes to addition of absorbent solution, sometimes to both. In the sense now used, c indicates the total correction, physical and chemical, for the reagents used.

tain its diameter sharply to the surface of the stopper. If the bore is widened out funnel-like next the stopper, traps are formed in which small gas

TABLE 28

FACTORS FOR CALCULATION OF CO_2 CONTENT OF BLOOD (FROM VAN SLYKE AND SENDROY (62))

TEMPERATURE	FACTORS BY WHICH MILLIMETERS P_{CO_2} ARE MULTIPLIED TO GIVE:									
	Millimoles CO_2 per liter of blood					Vol. per cent CO_2 in blood				
	Sample = 0.2 cc.	Sample = 1.0 cc.				Sample = 0.2 cc	Sample = 1.0 cc.			
		S = 3.5 cc.		S = 7.0 cc.			S = 3.5 cc.		S = 7.0 cc.	
	S = 2.0 cc. a = 0.5 cc. i = 1.037	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017	S = 2.0 cc. a = 0.5 cc. i = 1.037	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017
°C.										
15	0.1514	0.0313	0.1229	0.0341	0.1335	0.3370	0.0697	0.2735	0.0758	0.2974
16	07	11	22	38	25	54	93	19	52	50
17	0.1499	10	15	35	15	38	89	04	46	28
18	92	08	08	33	06	22	86	0.2690	41	06
19	86	06	02	31	0.1297	07	82	75	36	0.2886
20	79	05	0.1196	28	88	0.3292	78	62	31	66
21	72	03	90	26	79	78	75	48	26	48
22	66	02	83	24	70	63	71	34	21	28
23	59	00	77	22	62	48	68	20	16	08
24	53	0.0299	71	19	53	34	65	07	11	0.2790
25	46	97	65	17	45	20	61	0.2594	07	72
26	40	96	60	15	37	06	58	81	02	53
27	34	94	54	13	29	0.3193	55	69	0.0698	36
28	28	93	49	11	22	79	52	57	93	20
29	22	91	43	10	15	66	49	45	89	04
30	16	90	38	08	08	53	46	33	85	0.2688
31	11	89	33	06	01	40	43	22	82	74
32	05	88	28	05	0.1195	28	40	11	78	59
33	00	86	23	03	88	15	37	00	74	44
34	0.1394	85	18	01	82	03	34	0.2489	71	30

To obtain factors for a sample other than 1 cc., divide the above factors for 1 cc. by the cubic centimeters of sample analyzed: e.g., for a 2-cc. sample, S, A, and a being the same, the factors are one-half of those for a 1-cc. sample.

bubbles are likely to be held back, sufficient to affect significantly the results of a micro analysis, such as is performed on blood filtrates. The 120-degree

angle in the bore of the cock must be exact, so that in all of its three positions the cock will unite the connected capillaries into smoothly continuous tubes. The rubber-capped outlet at the bottom of the tube connecting the two bulbs is not essential. Though convenient in cleaning the pipette, it makes a point of structural weakness.

Calculations

The calculations are simpler than those with the volumetric apparatus, for the reason that the barometric pressure plays no part in them. The observed partial pressure of each gas is multiplied by a single factor, dependent on the temperature. These factors are given in tables for the different analyses described in the following pages.

The factors used are calculated by the following formulas, which are derived from equation 1, given on p. 249 in the description of CO_2 determination by the volumetric apparatus.

In place of the pressure, $B-W$, in equation 1, the observed pressure P is substituted and in place of V_t, the fixed gas volume a. This gives

$$(2) \qquad V_{0°, 760} = P \times \frac{i\ a}{760\ (1 + 0.00384\ t)} \times \left(1 + \frac{S\ \alpha'}{A - S}\right).$$

To correct for the effect of temperature in expanding mercury more than glass, Van Slyke and Neill changed the coefficient of t from 0.00367 to 0.00384. A factor, $\frac{100}{\text{cc. blood sample}}$ is introduced, to convert $V_{0°,760}$ to terms of volumes per cent of gas in blood. Inclusion of this factor gives

$$(3) \qquad \text{Volume per cent of any gas in blood} = P \times \left[\frac{0.1316\ i\ a}{(\text{cc. sample})} \times \frac{1}{(1 + 0.00384\ t)} \times \left(1 + \frac{S\ \alpha'}{A - S}\right)\right]$$
$$= P \times \text{volume per cent factor}$$

To express results in millimoles instead of cubic centimeters of gas, the right hand member of equation 3 is divided by the number of cubic centimeters of gas, measured at 0°, 760 mm., that constitute 1 milligram molecule. This, for a perfect gas, is 22.4 cc., a number which is approximated within the limit of error by O_2, N_2 and CO. Hence, for O_2, CO and N_2

$$(4) \quad \text{Millimoles of gas in sample} = P \times \frac{i\ a}{17{,}024\ (1 + 0.00384\ t)} \times \left(1 + \frac{S\ \alpha'}{A - S}\right)$$

$$\text{(5)} \quad \text{Millimoles of } O_2, CO, \text{ or } N_2 \text{ per liter of blood} = P \times \left| \frac{0.0587\, i\, a}{(\text{cc. sample})} \times \frac{1}{1 + 0.00384\, t} \times \right.$$

$$1 + \frac{S\, a}{A - S}$$

$$= P \times \text{mM. per liter factor.}$$

For CO_2 Guye and Pinza (13) have found that a milligram molecule of the gas at 0°, 760 mm. occupies a slightly smaller volume, 22.26 instead of 22.40 cc. Hence the numerical factor 0.00587 in equation 5 becomes changed to 0.00591 for CO_2.

The value of the CO_2 reabsorption factor, i, for the manometric apparatus was found by Van Slyke and Sendroy (62) to be 1.017 when the gas was brought to a volume of 2 cc., 1.037 when it was brought to a volume of 0.5 cc.

In case an apparatus is used with values of a differing significantly from 0.5 and 2.0 cc., the factors given in the table which applies to any analysis done may be corrected by multiplying them all by the ratio,

$$\frac{\text{actual } a}{0.500} \text{ or } \frac{\text{actual } a}{2.000}$$

For example, if a chamber on calibration is found to have an a value of 2.010 instead of 2.000 cc., the factors in whatever table applies to the analysis calculated, are all multiplied by $\frac{2.010}{2.000}$, or 1.005. Or, instead of preparing a new table, the factors in the printed table may be used, and the result may be multiplied by the correction factor, 1.005 in the above example. When many analyses are to be done it is more convenient to prepare a corrected table.

Manometric Determinations of the Blood Gases

CARBON DIOXIDE IN PLASMA OR WHOLE BLOOD. VAN SLYKE AND NEILL (59)

Reagents

0.1 N or 0.01 N lactic acid.

1 N sodium hydroxide solution, air-free.

5 N sodium hydroxide.

Caprylic alcohol.

These have been described under "General reagents."

Procedure for CO_2 in 1-cc. samples

After the extraction chamber has been cleaned (p. 236) a drop of caprylic alcohol is drawn into the capillary above the cock at the top.

2.5 cc. of CO_2-free 0.1 N lactic acid are placed in the cup. The sample of 1 cc. of blood or plasma is then run, from a rubber tipped pipette, into the chamber, (see fig. 29 or 30) and the 2.5 cc. of 0.1 N lactic acid are run in after the blood sample, making a total volume of 3.5 cc. of fluid in the chamber and the capillary connecting it with the cup. The cock is sealed with mercury run in from the cup and the apparatus is evacuated by lowering the leveling bulb until the mercury in the chamber has fallen to the 50-cc. mark. The cock leading to the leveling bulb is closed and the reaction mixture is shaken two minutes.

When extraction of the gas is complete, mercury is readmitted, with the precautions previously discussed for carbon dioxide determinations under "Adjustment of gas volume," until the gas volume in the chamber has been reduced to 2 cc. The manometer is then read (p_1 mm.).

With whole blood the absorption of carbon dioxide with 1.0 cc. of air-free 1 N sodium hydroxide is performed as follows: Mercury is admitted from the leveling bulb into the chamber of the apparatus until the gas volume in the chamber has been reduced to about 5 cc. The 4-mm. cock connecting chamber and leveling bulb is then closed. Two cc. of air-free 1 N sodium hydroxide solution are placed in the cup of the chamber. One cc. of the alkali solution is run slowly into the partially evacuated space to absorb the carbon dioxide. Absorption will be complete if as much as 30 seconds is taken to run in the alkali. If the O_2-CO_2 mixture in the chamber were at atmospheric pressure when the air-free alkali solution was added, the latter would absorb, by physical solution, a little O_2 along with the CO_2 removed by combination with the alkali. After the alkali has been added, a few drops of drops of mercury are admitted from the cup to dislodge the drop of alkali that adheres under the cock at the top of the chamber. Use, with whole blood, of an alkali stronger than 1 N is undesirable, because it would cause a gummy precipitate of hemoglobin.

With plasma or water solutions of carbonates a simpler absorption technique may be used. The carbon dioxide may be absorbed with 0.2 cc. of 5 N sodium hydroxide measured with a pipette into the cup of the apparatus. This solution has solubility coefficients for gases only one-tenth those of water and may be used without being previously freed of air. It is added with the contents of the chamber at but slight negative pressure, with the cock to the leveling bulb open. When the carbon dioxide is absorbed the diluted plasma solution rises and washes the concentrated alkali out of the upper tube of the chamber with sufficient completeness to prevent error from vapor tension lowering.

The c correction with the addition of so little hydroxide is nearly negligible. This technique can not be used with whole blood. In the case of whole blood, after absorption of CO_2 there is so much oxygen left in the top of the chamber that the blood solution would not rise high enough to wash out the concentrated alkali. The latter would consequently lower the manometer reading, p_2, 2 or 3 mm. by lowering the vapor tension in the top of the chamber.

Absorption of the carbon dioxide having been completed, the solution in the chamber is lowered until its surface is below the 2 cc. mark. The leveling bulb is placed at the middle level as in figures 36 and 37, and mercury is readmitted until the meniscus of the solution is again on the 2-cc. a mark. The p_2 reading is then noted on the manometer. The pressure P_{CO_2} of carbon dioxide is

$$P_{CO_2} = p_1 - p_2 - c$$

where c is the correction for the effect of the introduction of the sodium hydroxide solution on the level of the mercury surface in the chamber.

The c correction for the apparatus is determined by a blank analysis in which 3.5 cc. of 0.1 N lactic acid are extracted two minutes in the apparatus, the manometer being read before and after the addition of 1.0 cc. of 1 N sodium hydroxide or 0.2 cc. of 5 N, as described above. The difference between the readings before and after addition of the hydroxide is the c correction.

Procedure for CO_2 in blood samples of 0.2 or 0.1 cc.

Three cubic centimeters of 0.01 N lactic acid and a drop of caprylic alcohol are freed of air and CO_2 by extracting for one minute in the evacuated chamber of the apparatus. All but about 0.5 cc. is then run up into the cup of the chamber.

The sample is then delivered, either "from rubber tipped pipette without stop-cock" as described on page 241 or "from pipette without either rubber tip or stop-cock" as described on page 242. After the delivery of the blood sample enough of the lactic acid from the cup is run into the chamber to bring the volume of solution there to 2 cc. The chamber is then evacuated till the mercury meniscus is lowered to the 50-cc. mark, and is shaken for 2 minutes.

Mercury is then admitted to the chamber, with the precautions outlined for "Adjustment of gas volumes. CO_2 analyses" on page 277, and the gas volume is brought to 0.5 cc. If whole blood is being analyzed the use of a frosted light behind the chamber to outline the red

meniscus is essential for accurate results. The reading p_1 is taken, a lens being used to increase the accuracy of reading both the meniscus and the manometer.

The cock to the leveling bulb is then opened, so that the contents of the chamber are under but slight negative pressure, and the gas collects in a small bubble under the cock of the chamber. 0.2 cc. of 5 N sodium hydroxide solution is measured with a pipette into the cup of the chamber, and is then slowly admitted to the chamber, until only enough remains above it to fill the capillary above the cock. The absorption of CO_2 is almost instantaneous. A few drops of mercury are run through the cock. The meniscus is returned to the 0.5-cc. mark and the p_2 reading is taken.

The CO_2 pressure, P_{CO_2} is calculated as

$$P_{CO_2} = p_1 - p_2 - c.$$

The c correction is obtained by performing a blank analysis, in which no blood is added. The manometer difference, $p_1 - p_2$, obtained in the blank analysis is the c correction.

Calculation of blood or plasma CO_2 content

The P_{CO_2} value is multiplied by the proper factor from table 28 to obtain the CO_2 content of the blood or serum. In case the sample is only 0.1 cc. the factor is found by doubling the factor given for 0.2 cc. samples.

Example. In analysis of a 1 cc. sample of plasma, p_1 = 345.2 mm., p_2 = 127.8 mm.; c = 0.5 mm., temperature of gas chamber = 21°. P_{CO_2} = 345.2 − 127.8 − 0.5 = 216.9 mm. From the fourth column of table 28 we find that the millimolar factor for 21° is 0.1190. Hence mM. CO_2 per liter plasma = 0.1190 × 216.9 = 25.81.

Graphic estimation of plasma CO_2 from whole blood CO_2

A. Estimation by the line chart of figure 39. In plasma the CO_2 content is about 1.7 times that in the cells. Consequently acidotic blood may show an ordinary normal CO_2 content if at the same time the blood is sufficiently anemic. To avoid such fallacies it is now customary in acid-base studies to analyze plasma. However, analysis of whole blood instead of plasma is sometimes necessary, when the supply of blood is limited or facilities are not available for separating the plasma without exposure to air. In such a case Van Slyke and Sendroy (63a) have shown that the CO_2 content of the plasma can be estimated from that of the whole blood with sufficient accuracy for most acid-base studies (within 1 or 2 volumes per cent) by means of the line chart in figure 39. In this figure a straight line, drawn from any

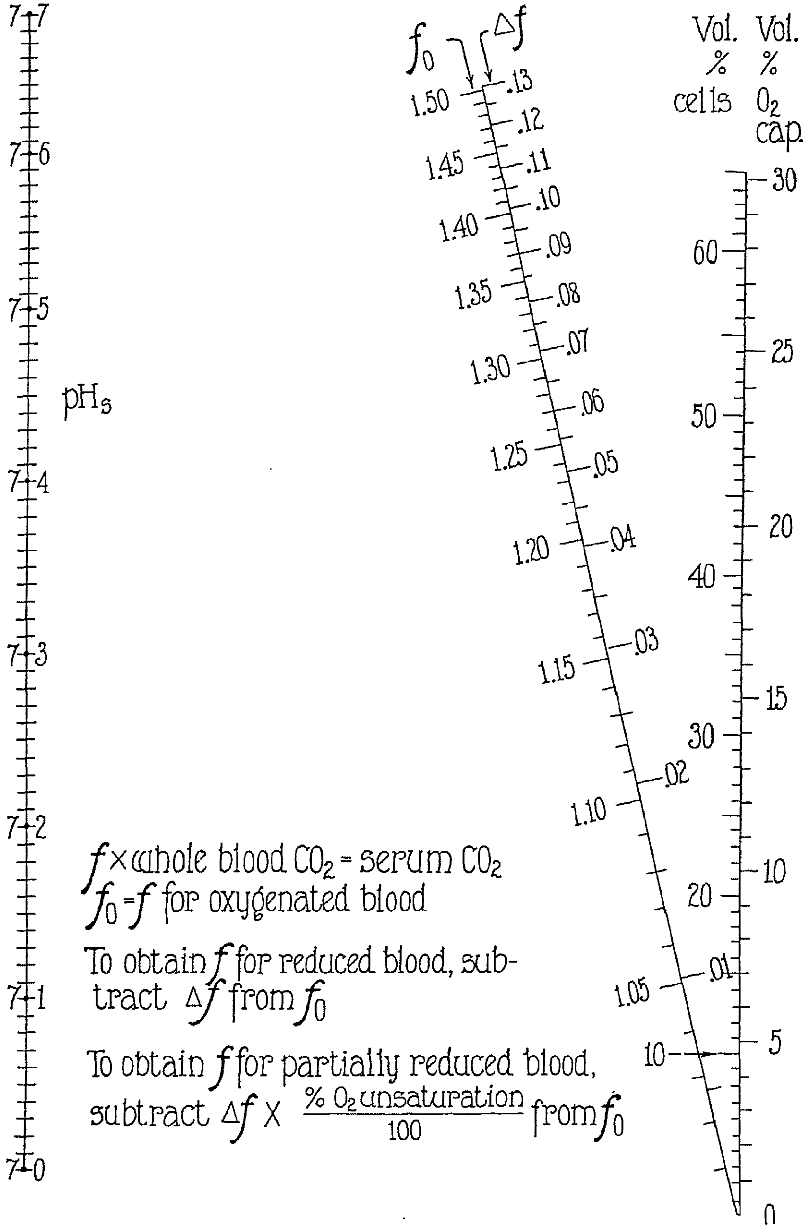


FIG. 39. Line chart for calculating plasma CO_2 content from whole blood CO_2 content. From Van Slyke and Sendroy (63a).

point on the pH_s scale through a point on the cell volume or oxygen capacity scale, intersects the slanting f_o line at a point which indicates the factor, f_o, by which the CO_2 content of *oxygenated* whole blood must be multiplied in order to obtain the serum or plasma CO_2 content.

If the hemoglobin of the blood is *completely reduced*, the interesected value of Δf, indicated on the scale to the right of the slanting line, is subtracted from the value of f_o for oxygenated blood, in order to obtain the value for reduced blood.

If, as in venous blood, the hemoglobin is *partly reduced*, the value subtracted from f_o is

$$\Delta f \times \frac{O_2 \text{ capacity} - O_2 \text{ content}}{O_2 \text{ capacity}}$$

Figure 39 can be used when either the hemoglobin content (oxygen capacity) or the cell volume content of the blood is known, as the right hand scale is graduated in terms of both values.

For many purposes for which knowledge of the plasma CO_2 content is desired in acid-base studies, estimation of the plasma CO_2 from whole blood CO_2 can be made with sufficient accuracy even when the pH_s and the degree of oxygenation of the blood are not known. The value of f is not much affected by changes in plasma reaction of less than 0.1 pH. If the blood is taken from a subject without definite alkalosis or acidosis, one may assume, for use with figure 39, that the pH_s is 7.40, without introducing into the estimated plasma CO_2 an error greater than 0.3 volume per cent. The change from complete oxidation to complete reduction of blood lowers the value of f by about 4 per cent. For arterial blood, usually 95 per cent saturated with oxygen, the uncorrected f_o may be used without significant error, except in cyanotic subjects. For venous blood, if one assumes 50 per cent oxygenation, the maximum possible error in f is 2 per cent, sufficient to cause an error in the estimated plasma CO_2 of about 1 volume per cent. In practice the error, caused by assuming 50 per cent oxygenation of venous blood, would rarely exceed 0.5 volume per cent of plasma CO_2.

B. Estimation by the line chart of figure 40. Figure 40, compiled by Sendroy (63*c*) is used in every respect like figure 39, except that the CO_2 tension, represented in the left hand scale, is used instead of pH_s. Figure 40 can be used in cases where the CO_2 tension is known, but not the pH_s. Theoretically the CO_2 tension is less directly related to the ratio of CO_2 contents in cells and plasma, but actually, *except* in cases of marked acidosis or alkalosis, figure 40 is about as exact as 39.

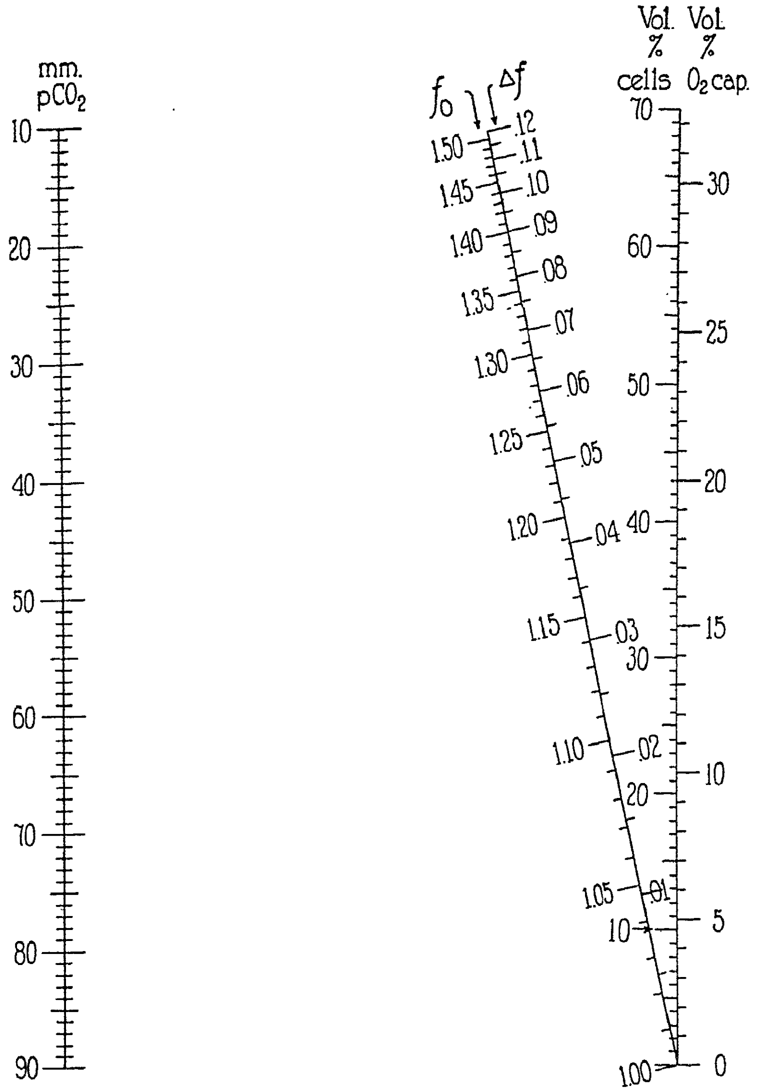


FIG. 40. Line chart for calculating plasma CO_2 content from whole blood CO_2 content. Same as figure 39, except that CO_2 tension is here used in place of pH_s in left hand scale. From Van Slyke, Sendroy, and Liu (63c).

CARBON DIOXIDE IN URINE

The determination of carbon dioxide in urine is performed in the same manner described above for carbon dioxide in blood. In analysis of urine, however, samples of varying sizes are taken, because, as shown by Marshall (21*c*) and Gamble (11*a*), alkaline urines contain immensely greater amounts of bicarbonate, and therefore of total CO_2, than do acid ones.[7] Gamble found that the free H_2CO_3 was fairly constant, averaging about 4.2 volumes per cent, while the bicarbonate increased with the pH according to the Henderson-Hasselbalch equation. The latter may be expressed in the form

$$\text{Log BHCO}_3 = \log \text{H}_2\text{CO}_3 + \text{pH} - \text{pK}',$$

the numerical value 4.2 volumes per cent or 1.9 millimoles being inserted for H_2CO_3 and 6.1 for pK′.

The relationship between pH, CO_2 content, and size of urine sample for analysis is the following:

pH up to 6.5, CO_2 5 to 12 volumes per cent, samples of 5 cc.

pH 6.5 to 7.5, CO_2 12 to 100 volumes per cent, samples of 1 cc.

pH above 7.5, CO_2 above 100 volumes per cent, samples of 0.2 cc.

If the pH is not known, one may take a 5-cc. sample. Henderson and Palmer (17*a*) found that 80 per cent of normal urines have pH values not exceeding 6.5, and therefore, according to the above rule, CO_2 contents suitable for 5-cc. samples. If the CO_2 obtained from 5 cc. of urine proves to be too much to measure, the analysis is repeated with a sample of 1 cc. or less.

Reagents

0.1 N hydrochloric acid.

5 N sodium hydroxide, described on page 233 under "General reagents."

Caprylic alcohol.

Procedure

A drop of octyl alcohol is run into the capillary at the top of the manometric chamber. Enough 0.1 N hydrochloric acid is placed in the cup of the manometric chamber to make the volume of acid plus urine

[7] Mainzer (21a), in order to avoid the necessity of frequently taking small samples of urine, employs a manometric chamber on which below the usual 2-cc. mark, two other bulbs of 2-cc. capacity are blown, so that the chamber can be calibrated, not only at 0.5 and 2 cc., but also at 4 and 6 cc. With this chamber 5-cc. samples can be analyzed of urine containing up to 50 volume per cent of CO_2, a content which ordinarily corresponds to a urinary pH of about 7.0.

sample 7 cc. The urine sample is then delivered into the chamber as described before in connection with figure 29 or 30 on page 240, and is followed into the chamber by the acid. The CO_2 is extracted by three minutes shaking, and p_1 is measured as in blood CO_2 analyses, with the gas volume at 2 cc. The CO_2 is absorbed with 0.3 cc. of 5 N

TABLE 29

FACTORS FOR CALCULATION OF CO_2 CONTENT OF URINE*

TEMPERATURE	FACTORS BY WHICH MILLIMETERS P_{CO_2} ARE MULTIPLIED TO GIVE					
	Millimoles CO_2 per liter urine			Volume per cent CO_2 in urine		
	Sample of 5 cc.	Sample of 1 cc.	Sample of 0.2 cc.	Sample of 5 cc.	Sample of 1 cc.	Sample of 0.2 cc.
°C.						
15	0.02670	0.1335	0.668	0.0595	0.2974	1.487
16	50	25	63	90	50	75
17	30	15	58	86	28	64
18	12	06	53	81	06	53
19	2594	1297	49	77	2886	43
20	76	88	44	73	66	33
21	58	79	40	70	48	24
22	40	70	35	66	28	14
23	24	62	31	64	08	04
24	06	53	27	60	2790	1.395
25	2490	45	23	54	72	86
26	74	37	19	51	53	76
27	58	29	15	47	36	68
28	44	22	11	44	20	60
29	30	15	08	41	04	52
30	16	08	04	38	2688	44
31	02	01	01	35	74	37
32	2390	1195	598	32	59	30
33	76	88	94	29	44	22
34	64	82	91	26	30	15

* All factors are calculated for conditions that S = 7 cc., a = 2 cc., and i = 1.017.

sodium hydroxide, and the alkali is mixed with the urine solution by raising and lowering the latter two or three times in the upper part of the chamber. Otherwise the heavy alkali solution might run under the urine solution, leaving the upper portions of the latter acid, so that some CO_2 might escape again to the gas phase. The reading p_2 is

taken after absorption of the CO_2, and the CO_2 pressure, P_{CO_2}, is calculated as

$$P_{CO_2} = p_1 - p_2 - c$$

The correction c is determined by a blank analysis in which 7 cc. of the 0.1 N HCl replace the acid + urine.

Calculation

The CO_2 content of the urine is obtained by multiplying P_{CO_2} by the proper factor from table 29.

BICARBONATE OF PLASMA AND URINE ESTIMATED FROM CO_2 CONTENT

Principles

The bicarbonate content can be calculated by means of the Henderson-Hasselbalch equation (see pp. 874–884 of volume I) from *the CO_2 content and the pH* by arranging the equation in the following form:

$$[BHCO_3] = [CO_2] \frac{1}{1 + \frac{[H^+]}{K'}} = [CO_2] \frac{1}{1 + \text{antilog } (pK' - pH)}$$

where $[CO_2]$ represents the total CO_2 content. The value of pK′ for blood serum has been found to be 6.10 (17). For urine, Mainzer (21b) has found, as might be expected from the varying composition of this fluid, that the value of pK′ showed marked fluctuations, covering as wide a range as 5.8 to 6.3. The average, however, was 6.1, the same as in serum.

From *CO_2 content and CO_2 tension* the $BHCO_3$ can be calculated by subtracting from the total CO_2 the part which is in solution as free carbonic acid. This is a mixture of anhydrous CO_2 and H_2CO_3, but is customarily for simplicity formulated as H_2CO_3. From the laws of gas solubility H_2CO_3 is calculated from the CO_2 tension, p as

$$\text{Millimoles } H_2CO_3 \text{ per liter} = \frac{\alpha\, p}{760 \times 0.02226}$$

$$= 0.0591\ \alpha\, p$$

$$= 0.0301\ p$$

where α represents the solubility coefficient of CO_2 in serum or urine. For normal serum at 38° Van Slyke, Sendroy, Hastings, and Neill (63b) found that the average value of α was 0.510. Substituting this value in place of α

gives 0.0301 p as the simple formula for calculating the H_2CO_3 of serum from the CO_2 tension of the blood.

The CO_2 tension of the arterial blood may be assumed to be the same as "arterial CO_2 tension" determined in the alveolar air as described in chapter IV of this volume. In the venous blood from the arm, however, one can not assume that p is the same as the "venous CO_2 tension" determined in the alveolar air, because the blood from the particular vein tapped may have a CO_2 tension considerably above or below that of the mixed venous blood from the right heart which determines the "venous CO_2 tension" found by the methods in chapter IV. The CO_2 tension of blood can be determined by the methods described on pages 298 and 309.

For urine Mainzer (21b) has found that the value of α at 38° varies from 0.441 to 0.514. For urine CO_2 tension see page 316.

Bicarbonate in blood plasma

A. By formula. If we substitute 6.10 in place of pK′ in the first of the above formulae we obtain

$$[BHCO_3] = [CO_2] \times \frac{1}{1 + \text{antilog}\ (6.10 - \text{pH})}$$

for use *when pH is used* to estimate the proportion of total CO_2 that is in the form of bicarbonate.

From the second of the preceding formulae we obtain, for use *when CO_2 tension, p, is used:*

$$\text{Millimoles } BHCO_3 = \text{Millimoles } CO_2 - 0.0301\ p$$

in which both $BHCO_3$ and CO_2 are expressed in millimoles per liter of serum. If the data, instead of millimoles, are in terms of volumes per cent of CO_2 factor 0.067 replaces 0.0301 as the coefficient of p.

B. By line chart. Figure 41 contains a line chart devised by Van Slyke and Sendroy (63a) by means of which serum or plasma $BHCO_3$ can be calculated graphically from the total CO_2 together with either pH_s or CO_2 tension. A straight line from the point indicating the observed CO_2 content to the point indicating either the observed pH_s or CO_2 tension, cuts the $BHCO_3$ scale at the point showing the $BHCO_3$ content in millimoles per liter.

Bicarbonate in urine

For determining the $BHCO_3$ in urine from the CO_2 content and pH the same formula as for serum may be used. It is less exact for urine because

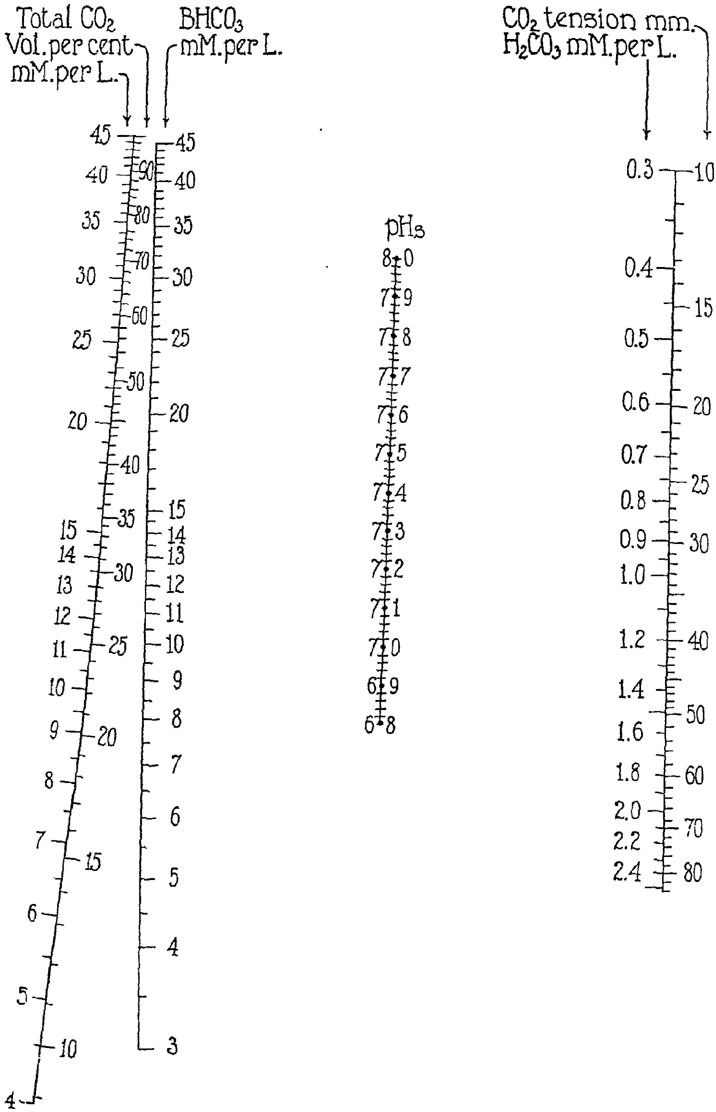


FIG. 41. Line chart for graphic calculation of pH, CO_2 tension, $BHCO_3$, etc., in blood serum. A straight line across any two scales cuts the other scales at points indicating simultaneously occurring values.

The chart is based on the Henderson-Hasselbalch equation, with values for CO_2 solubility in serum from Van Slyke, Sendroy, Hastings, and Neill (63b) and for pK from Hastings, Sendroy, and Van Slyke (17). Chart from Van Slyke and Sendroy (63a).

of the fact that the values of α and pK′ used as constants are subject to considerable variation in urine.

A. By multiplying total CO_2 by the per cent present as $BHCO_3$, indicated by the pH.

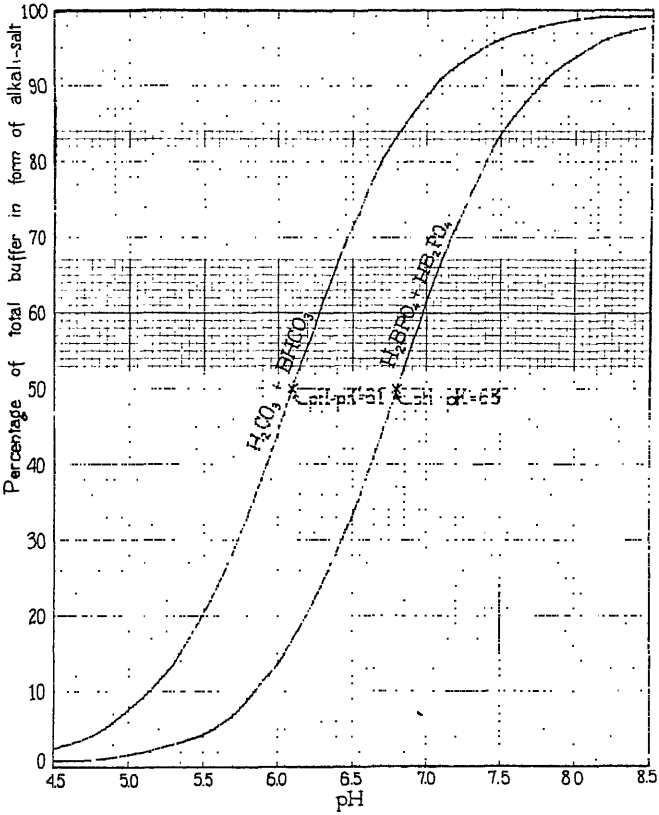


FIG. 42. Curves showing the proportion of carbonic acid in the form of $BHCO_3$ and of phosphate in the form of B_2HPO_4 at varying pH. $BHCO_3$ curve applies to blood serum, and approximately to urine. Phosphate curve is for Sorensen M/15 phosphate buffer solutions.

The most practicable general procedure is to estimate from the ordinate of the H_2CO_3-$BHCO_3$ curve in figure 42 the per cent of total CO_2 that is in the form of $BHCO_3$, and multiply the total CO_2 content by the per cent, e.g., if the CO_2 content of the urine is 7.0 mM. per liter and the pH is 6.5, the curve

indicates that 71.5 per cent of the CO_2, or $0.715 \times 7.0 = 5.0$ millimoles of $BHCO_3$ per liter is present.

Procedure *A* applies to either normal or pathological urines.

B. By subtracting the mean H_2CO_3 of normal urine.

Gamble (11a) found that in normal urine the H_2CO_3 content is fairly constant at 4.2 volumes per cent, or 1.9 millimoles per liter. Consequently subtracting 4.2 volumes per cent or 1.9 mM. per liter, from the total CO_2 gives the bicarbonate. E.g., in the same urine specified in the above example, subtracting 1.9 mM. from the 7.0 mM. total CO_2 leaves 5.1 mM. $BHCO_3$. This procedure assumes a constant CO_2 tension in urine and is therefore less accurate than procedure *A*. It suffices, however, when one wishes to estimate amounts of $BHCO_3$ such as are found in urines of pH greater than 7.0, where H_2CO_3 is small in comparsion with $BHCO_3$.

PLASMA CARBON DIOXIDE COMBINING CAPACITY OF VAN SLYKE AND CULLEN (53)

The applicability of this determination as a measure of the alkaline reserve in conditions not complicated by respiratory abnormality has been discussed on page 251.

The technique for drawing and centrifuging the blood, and for storing the plasma and saturating it with air containing 5.5 per cent of CO_2, is the same as described on page 253 for preparing the plasma for analysis by the volumetric apparatus.

Reagents

0.1 N lactic acid, CO_2-free.
Caprylic alcohol.

Procedure

For the analysis a drop of octyl alcohol is drawn into the capillary below the cup of the manometric chamber, and 1.5 cc. of 0.1 N lactic acid are measured into the cup. The 1 cc. sample of plasma, previously saturated with air containing 5.5 per cent of CO_2, is delivered into the chamber by the technique for "delivery of blood sample from pipette without stop-cock" described on page 241, and illustrated in figure 29. The 1.5 cc. of lactic acid is run in after the plasma, the cock is sealed with mercury, and the chamber is evacuated and shaken two minutes.

The volume of gas is reduced to 2 cc. with the precautions described on p. 277 for carbon dioxide determinations, and p_1 is read on the manometer. The gas is then ejected without loss of solution, as described on page 279, and the meniscus of the solution is returned to

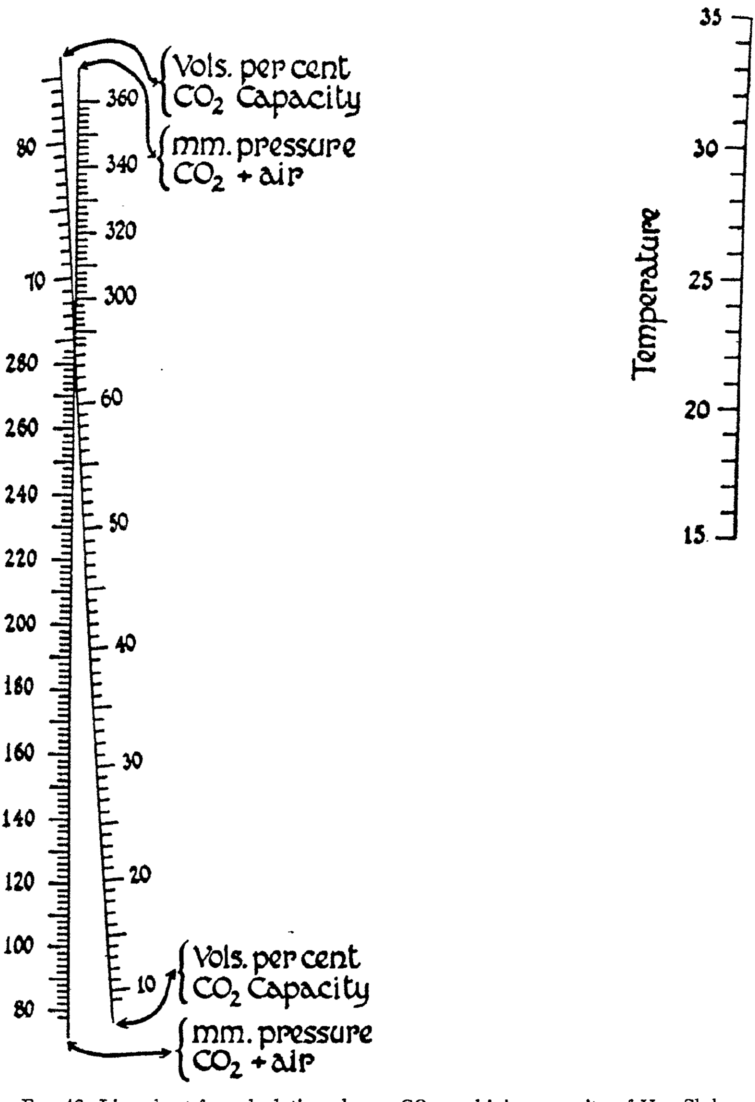


FIG. 43. Line chart for calculating plasma CO_2 combining capacity of Van Slyke and Cullen from manometric readings. A straight line intersecting the "temperature" and "mm pressure CO_2 + air" scales at points of observed values cuts the "vols. per cent CO_2 capacity" scale at the point showing the CO_2 capacity of the plasma, when the plasma sample analyzed is 1 cc. From Van Slyke and Neill (59).

the 2-cc. mark in the gas free chamber. The zero pressure p_0 is then read on the manometer.

The pressure of the CO_2 + air extracted from the 2.5 cc. of solution is $p_1 - p_0$ mm. From the pressure thus obtained and the temperature the CO_2 capacity of Van Slyke and Cullen is calculated graphically by means of the line chart in figure 43.

Micro determination

In a 50-cc. separatory funnel enough plasma is saturated with air containing 5.5 per cent CO_2 so that one or preferably two samples of 0.2 cc. can be obtained. For the analysis a drop of octyl alcohol and 0.2 cc. of the saturated plasma are run into the chamber of the apparatus followed by enough 0.01 N lactic acid to bring the volume of solution in the chamber down to the 2.0 cc. mark. The reading of the pressure of the extracted gases is made at 0.5 cc. volume. The pressure observed is applied to the line chart of figure 43, as in calculating the results of the macro determination. The result is multiplied by 1.26 to give the correct capacity by the micro method.

The sample in the micro analysis is 0.2 as great as in the macro analysis, and the volume at which the gas pressure is measured is 0.25 as great. Hence the pressure observed in the micro analysis of a given plasma would be only 0.20/0.25, or 1/1.25 as great as in the macro analysis, if the corrections for unextracted and redissolved CO_2 were the same. However, with the smaller volume of solution (2 cc. vs. 2.5 cc.) in the chamber, extraction is more complete. On the other hand, reabsorption of CO_2 in the microanalysis, when the gas volume is reduced to 0.5 cc., is 3.7 per cent compared with 1.7 per cent in the macro analysis. The effects are opposite in direction, and their difference suffices to raise the factor from 1.25 to 1.26.

EISENMAN'S INTERPOLATION METHOD FOR DETERMINATION OF SERUM pH AND CO_2 TENSION (8A)

Principle

The CO_2 absorption curve of the serum or plasma is located, and the point representing the value of the CO_2 content of the anaerobically centrifuged serum or plasma is interpolated on the curve. Thereby the CO_2 tension of the blood as drawn and the pH of the plasma can be ascertained.

Three samples of serum or plasma are analyzed for CO_2 content: one at once in the condition in which it is separated anaerobically from the cells, and the other two after equilibration at 38° with air containing CO_2 at known tensions below and above that of the circulating blood. Ordinarily air with

CO_2 tensions of about 30 and 60 mm. is used for the saturation. The results, in terms of CO_2 tension of the air used and of CO_2 content found in the two saturated plasma samples, are plotted as points A and B on Peters' logarithmic chart, as shown in figure 44 (for discussion of this type of chart see "Logarithmically plotted CO_2 absorption curves" on pp. 913–914 of volume I). This type of chart has the advantage that CO_2 absorption curves of serum or plasma (likewise those of whole blood) plotted on it are approximately straight lines: hence a linear "curve" may be located by determining two points and drawing a straight line through them. On this line is marked the point x indicating the CO_2 content of the serum sample analyzed as separated from the blood cells. The ordinates of this point indicate the CO_2 tension and pH_s of the blood as drawn.

The accuracy of the procedure for pH_s estimation may be deduced from equation 27 in the "Carbonic acid and acid-base balance" chapter of volume I. From this equation one calculates that, in the absence of cells, there is 1.8 volumes per cent change in the CO_2 content of normal separated serum per 0.1 change in pH_s. If the rectilinear form of the CO_2 absorption curve holds exactly, and the curve itself is located without error, a serum analysis accurate within 0.2 volumes per cent (about the limit of accuracy of the method) would yield a pH accurate within 0.01. However, the location of the line itself is subject to the errors of the two sets of analyses by which it is located, and the absorption curve is not necessarily an absolutely straight line, although it closely approaches one. If the height of the absorption curve, in terms of CO_2 content, is in error by 0.2 volume per cent, and the error is opposite in direction to a similar error in the analysis of the plasma as separated, the total error in pH from these sources would be 0.02. With good manometric analyses performed in duplicate on 1 cc. plasma or serum samples, results reproducible within 2 or 3 mm. of CO_2 tension and within 0.02 or 0.03 pH_s may be expected. When 0.2 cc. samples are used the error may be twice as great.

The accuracy of the constants, α and pK$'$, of the Hasselbalch equation by which the chart of figure 44 is plotted, sets the limit of the accuracy with which electrometric pH_s results can be checked by this gasometric method. The errors in α and pK$'$ are at present probably slight, since these constants have been determined with precise technique and many precautions (17, 63b).

When, however, the body temperature is much above or below 38°, these constants are altered, and likewise the level of the absorption curve. *The error of the method therefore increases to an extent not yet determined when it is used to estimate pH_s in febrile subjects.*

Drawing of blood and separation of serum or plasma

The blood is drawn with aerobic precautions, described in chapter II, to prevent loss of CO_2 or access of O_2. If an anticoagulant is added it must be one that is neutral and free from buffers. From the time the blood is

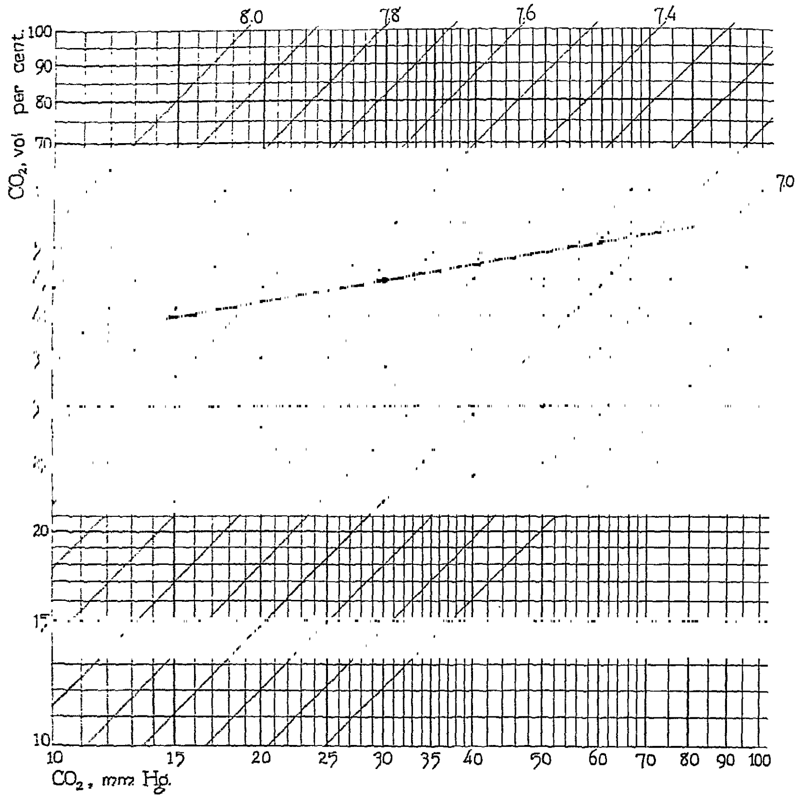


FIG. 44. Logarithmic pH chart for blood serum. The ordinates represent CO_2 contents of plasma in volumes per cent, and the abscissae CO_2 tensions in mm. of mercury. From Peters (28a).

drawn until, as soon as possible, the serum or plasma is separated, no change of CO_2 or O_2 content must be permitted to occur, either by contact with air or by allowing time enough for respiration of the cells to affect the gas content. Nor must lactic acid formation by glycolysis be given opportunity to occur. Such changes in the whole blood affect both the pH and bicarbonate

content of the plasma in the manner discussed in volume I on page 902 and under "Donnan equilibrium" on page 1020.

The separation of the serum or plasma from the cells is likewise carried out under anaerobic precautions, as described in chapter II.

Saturation of serum or plasma at 38° with air of known CO_2 tension

Various procedures may be used, differing chiefly in the manner in which quantitative mixtures of air and CO_2 are prepared. Two such procedures will be described. The first, introduced by Van Slyke, Wu, and McLean (65), is based on the measurement of gases into a tonometer by means of pressure read on a manometer. It is convenient, does not require measurement of the volume of the tonometer, and the calculation is made by a relatively simple formula.

Procedure II is an earlier one used by Austin, Cullen, Hastings, McLean, Peters, and Van Syke (3) before the convenience of the pressure method had been realized. It is based on the measurement of calculated volumes of CO_2 into tonometers of known volume. The technique itself is about as simple as that of the measurement by pressure, but the calculations are more complicated, and involve the barometric pressure and the volume of each tonometer used. We include a description of this procedure, however, because it is the one which Eisenman used.

I. ***Preparation of air-CO_2 mixture by pressure measurement.*** The ***apparatus*** shown in figure 45 is used for both procedures I and II. For I, however, the gas burette shown in the figure is omitted. The source of CO_2 is a Kipp generator containing marble chips and dilute hydrochloric acid (1 part of concentrated HCl to 2 parts of water). The CO_2 is bubbled through water in a wash bottle before it reaches cock *d*. The air inlet is through a mercury valve with a mercury column 80-mm. deep, so that when as much air as possible is admitted to the tonometer through this valve the pressure reached in the tonometer will be 80-mm. less than atmospheric. This difference is desirable in order to prevent danger of subsequent forcing out of a cock of the tonometer when the pressure of the contained gas is increased by raising the temperature to 38°. The bore of the manometer tube should be 4 to 5 mm., and the inner diameter of the bottle serving as mercury reservoir at the bottom of the manometer should be approximately 10 times the inner diameter of the manometer tube. Then the cross section of the bottle is 100 times that of the tube, and for each mm. rise of the mercury in the tube there is a fall of 0.01 mm. in the mer-

cury in the bottle. Hence 1 per cent can be added to any observed change in the manometer reading in order to obtain the actual pressure change in the system.

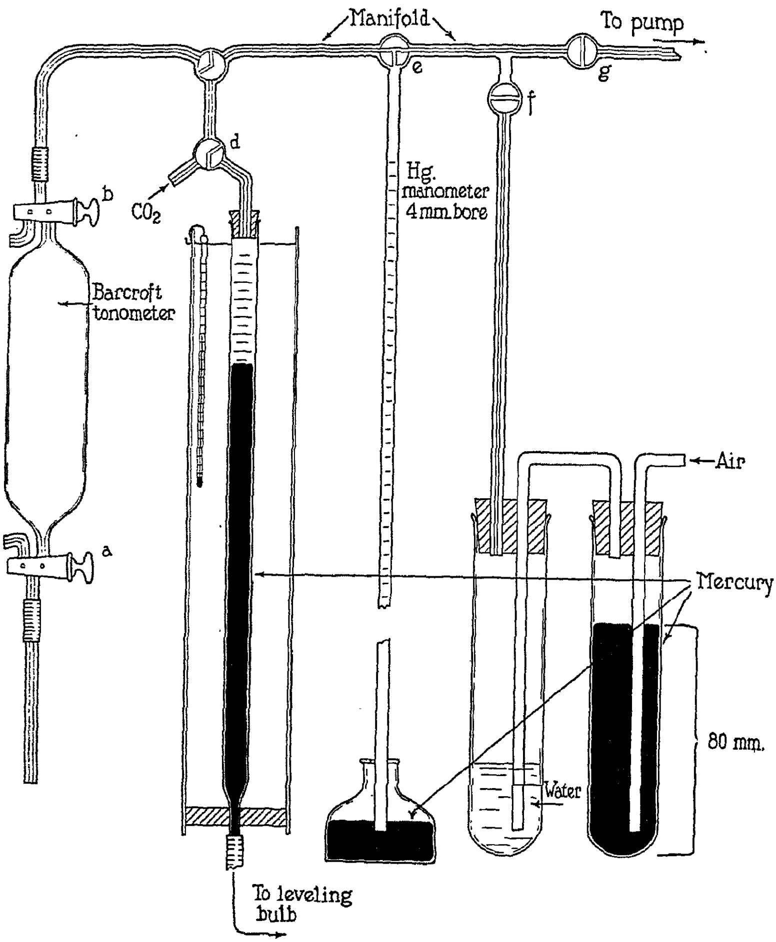


FIG. 45. Apparatus for filling vessel with gas mixture containing known tensions of the constituent gases. From Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (3).

The *procedure* is as follows. The tonometer is attached as shown in figure 45 and the system is evacuated until the column of mercury in the manometer has risen 200 or 300 mm. Cock *g* leading to the pump

is then closed, and the manometer is observed for a minute or two in order to see whether the system is free from leaks. If all connections are tight the manometer remains constant, and its reading is noted. Some CO_2 is then wasted through cock *d* so that air which may have diffused into the rubber connections leading to the Kipp generator may be replaced by pure CO_2. The waste CO_2 is passed out into the air through the limb of cock *d*. (Cock *d* in the figure is shown connected with the gas burette, which is omitted from the apparatus for the present use.) Then cock *d* is turned to admit CO_2 slowly into the manifold and tonometer. The admission is continued until the mercury in the manometer has fallen the desired distance. This distance is about 0.9 as many millimeters as represent the CO_2 tension desired at 38° in the tonometer. Therefore, for the tonometer that is to have at 38° approximately 30 mm. CO_2 tension the manometer drop should be about 27, and for the tonometer with 60 mm. the manometer fall should be about 56. When the desired amount of CO_2 has been admitted cock d is closed, the manometer is read accurately, and the room temperature is noted. Then through cock *f* as much air is admitted to the tonometer as will pass the mercury trap. The tonometer is then closed, disconnected from the manifold, and is rotated in a bath at 38° for fifteen minutes.

The *calculation* of the CO_2 tension in the tonometer after it has been warmed to 38° is made by equation 1:

$$p_{38^\circ} = 1.01\ p_t \times \frac{311}{273 + t} \qquad p_t \times \frac{314}{273 + t} \tag{1}$$

p_{38° = CO_2 tension in the tonometer at 38°.

p_t = CO_2 tension as measured at room temperature by the uncorrected manometer fall during the admission of CO_2.

t = room temperature in degrees Centigrade.

The factor 1.01 corrects, as mentioned above, for the rise of mercury in the manometer reservoir that accompanies the fall of mercury in the manometer tube. The factor, $\frac{311}{273 + t}$ corrects for the rise in CO_2 pressure, proportional to the rise in absolute temperature, that is caused by warming the tonometer from t° to 38°.

It is desirable that the tonometer should be as much as 30-fold the volume of the serum that is equilibrated. The serum will then approach the initial CO_2 tension closely enough to limit the error from this factor in the inter-

polated CO_2 tension to a fraction of a millimeter, and in the interpolated pH to the third decimal place, as may be deduced from the following paragraphs.

In figure 44 points A and B represent the CO_2 contents of samples of normal serum equilibrated at 38° with air containing CO_2 at 30 and 60 mm. tension respectively. Point x represents the CO_2 content of the serum as separated anaerobically from the fresh blood, and the interpolated position of x on the line A B indicates the CO_2 tension and pH of that blood.

Let us assume that 2 serum samples are each equilibrated with 30 volumes of air containing 30 and 60 mm. respectively of initial CO_2 tension, and that the initial CO_2 tension of the serum is intermediate at 45 mm. In this case one can calculate that the final CO_2 tensions reached at the end of equilibration by both serum and gas will be 31 and 59 mm. in the two tonometers. The points A and B (figure 44) will be approximately 0.25 volume per cent above and below their correct levels for 30 and 60 mm. CO_2 tension (As illustrated by the line A-B in figure 44, the CO_2 content of ordinary separated serum rises about 0.25 volume per cent per 1 mm. increase in CO_2 tension.) The two opposite errors at the ends of the line AB compensate each other in the middle of the line, so that at the point where x is interpolated the error is negligible. If the serum has a low initial CO_2 tension, so that x lies near A, point A will be located with almost exact precision, because there will be little interchange of CO_2 between serum and gas. The error in point B will be greater, but will have little effect on the level of x, because the latter is near the correct point, A. If x is located near B similar conditions will give an approximately exact result. Therefore, whether the CO_2 tension, x, of the drawn blood is near A or B or in the space midway between them, shift of CO_2 such as can occur between the serum and the gas phase during equilibration will cause no significant error in the interpolation of the position of x.

The change in CO_2 tension of points A or B (figure 44) that is caused by the absorption or giving off of CO_2 by the serum during the equilibration can be calculated by equation 2, which was developed by Austin et al (3).

$$p_f = p_i - \frac{8.66 \Delta V_S}{V_{Tn} - V_S} \qquad (2)$$

p_i is the initial value of $p_{38°}$ calculated by equation 1 on the assumption that the amount of CO_2 taken up or given off by the serum during equilibration is zero in its effect on the final CO_2 tension. p_f is the final tension actually reached in the tonometer at the end of equilibration. Δ is the volume per cent by which the serum CO_2 content *increases* during the equilibration (Δ is negative when the CO_2 content of the serum decreases, as it usually does when the serum is equilibrated at the lower CO_2 tension represented by point A in figure 44). V_{Tn} indicates the volume content of the tonometer, V_S the volume of serum in the tonometer. If, as recommended above, V_{Tn} is 30-fold V_S, absorption by the serum of 5 per cent of its volume of CO_2 during equilibration would change the CO_2 tension of the gas by

$$\frac{8.66 \times 5 \times 1}{29} = 1.5 \text{ mm.}$$

Equation 2 is derived as follows: The volume of CO_2 removed from the gas phase by absorption into the plasma is $V_S \times \frac{\Delta}{100}$, when the gas volume is expressed in cubic centimeters measured at 0°, 760 mm. In order to calculate the CO_2 volume at 38°, the above value must be multiplied by $\frac{311}{273}$. The pressure exerted by this amount of CO_2 in the tonometer gas space, $V_{Tn} - V_S$, is equal to the ratio, CO_2 volume: total gas space, in atmospheres, and to that ratio multiplied by 760 if the pressure is expressed in mm. of mercury. Hence the pressure correction to subtract from or add to p_i will be

$$\frac{\text{Cubic centimeters of } CO_2 \text{ absorbed (measured at 0°, 760 mm.)} \times \frac{311}{273}}{\text{Cubic centimeters of gas space in tonometer}} \times 760$$

$$= \frac{\frac{\Delta \times V_S}{100} \times \frac{311}{273}}{V_{Tn} - V_S} \times 760 = \frac{8.66\, V_S\, \Delta}{V_{Tn} - V_S}$$

II. Preparation of air-CO_2 mixture by volumetric measurement. The apparatus is used as shown in figure 45, with the gas burette in place. The tonometer and manifold are evacuated until the pressure is reduced to about half an atmosphere. Cocks *b* and *c* are then closed as shown in figure 45. CO_2 from the generator is then wasted through cockd *d*, *c*, and *b*, so that air which may have diffused into the CO_2 in the rubber connections leading to the generator is washed out, and the capillary tubes between *d* and *b* are filled with CO_2. Cocks *d* and *b* are then closed in the order given, and *d* is turned so that the gas burette is filled with CO_2. With cocks *c* and *d* turned as shown in the figure the mercury surface in the leveling bulb is brought even with that in the burette, and the reading is recorded of the burette with the CO_2 in it at atmospheric pressure. The temperature of the gas in the burrette is read from the thermometer in the water jacket and the barometric pressure is noted.

The volume of CO_2 required to bring the tonometer gas to the desired CO_2 tension is now calculated by means of equation 3.

$$V_{CO_2} = \frac{P\,(V_{Tn} - V_S)}{T_{Tn}} \times \frac{T_{CO_2}}{B - W_{CO_2}} \quad (3)$$

in which V_{CO_2}, T_{CO_2} and W_{CO_2} represent the volume, absolute temperature and vapor pressure, respectively, of the gas in the burette, B = the barometric pressure, V_{Tn} and V_S, = volumes of tonometer and serum

respectively, T_{Tn} = temperature at which the blood is to be equilibrated, P is the desired pressure of CO_2 at temperature T_{Tn}, which is usually 38°C., or 311 absolute. The first factor of this equation can be calculated in advance (it is for this reason that the equation is arranged in its otherwise illogical form). The vapor pressure, W_{CO_2}, corresponding to the observed temperature, T_{CO_2} is obtained by reference to table 18 on page 163.

Example

P = 40 mm. of CO_2 tension desired.

V_{Tn} = 61.5 cc. volume of tonometer between the two cocks.

V_B = 3.0 cc. of serum.

$T_{Tn} = 38° + 273° = 311°.$

$T_{CO_2} = 21° + 273° = 294°.$

B = 754 mm.

W_{CO_2} = 18.5 mm. vapor tension of water at 21°.

$$V_{CO_2} = \frac{40\ (61.5 - 3.0)}{311} \times \frac{294}{754 - 18.5} = \frac{40 \times 58.5 \times 294}{311 \times 735.5} = 3.01 \text{ cc. of } CO_2 \text{ gas.}$$

The desired volume of CO_2, V_{CO_2} is now introduced into the tonometer. To do this cocks *c* and *d* are turned to connect the burette with cock *b*, the mercury leveling bulb of the burette is raised so that the surface of mercury in the bulb is at a level slightly above that which will be taken by the mercury in the burette when the desired volume of gas has been delivered from it. Then cock *b* is turned so that the CO_2 is slowly admitted into the partly evacuated tonometer. When the desired volume has been transferred cock *b* is closed. At this moment the surface of the mercury in the leveling bulb should be even with that in the gas burette. In case exactly the desired amount of CO_2 has not been admitted to the tonometer, it is not necessary to repeat the procedure. The amount that has been admitted is measured, and the resultant P is calculated by rearranging equation 3 into the form of equation 4:

$$(4) \qquad P = \frac{V_{CO_2}}{V_{Tn} - V_S} \times \frac{T_{Tn}\ (B - W_{CO_2})}{T_{CO}}$$

The volume V_S of plasma or serum is measured into a test tube and thence is drawn up into the still partly evacuated tonometer through the capillary at the bottom and cock a. Enough air is permitted to follow the plasma to sweep all of it up into the tonometer, but not enough to fill the tonometer to atmospheric pressure.

The tonometer is finally connected through cocks b, c, and f with the air intake and air is allowed to enter as long as it will. The pressure in the tonometer is then 80 mm. below atmospheric. The tonometer is then closed and disconnected from the manifold.

Equilibration. After the tonometer has been filled by either procedure I or II the lower tube is wiped free of adherent serum with a pipe cleaner, and the ends of both tubes are covered by rubber caps. The tonometer is then rotated in a water bath at 38° for fifteen minutes in such a manner that the serum is distributed in an even film over the inner wall.

Sampling of saturated serum or plasma for analysis

After equilibration the tonometer is placed upright in the bath, so that one capillary outlet emerges. The rubber cap is removed from this and a rubber tube leading to a mercury bulb containing enough mercury to fill the tonometer is attached. A little mercury is wasted through the three-way cock. The tonometer is now inverted, still in the bath, so that the end attached to the mercury bulb is below. The upper cock of the tonometer is opened to the air, and mercury is slowly admitted through the lower cock until the serum rises to the upper cock, which is then closed. The tonometer is then removed from the bath. The lower cock is kept open, so that the leveling bulb remains connected with the interior of the tonometer, and the leveling bulb is elevated slightly so that the contents of the tonometer are under a little positive pressure. If both cocks of the tonometer were closed while the mercury and serum contracted during cooling to room temperature, a vacuum bubble would be formed and CO_2 would escape into it from the serum. A piece of rubber tubing about 2 cm. long is attached to the upper outlet of the tonometer, and samples of serum are drawn for analysis as described under "Measuring blood samples" on page 58 of chapter II. In the present case the tonometer is used in exactly the manner described for use of the mercury sample container there (fig. 6), the rubber tube being closed by a pinch clamp when a plasma sample is not being drawn.

Analysis of serum

The CO_2 contents of the original serum and of each of the saturated serum samples are determined separately by the usual manometeric procedures described on pages 283–286. Equilibration of 3-cc. samples of serum permits duplicate analyses of 1-cc. samples. The use of micro-technique, with smaller amounts and analysis of 0.2-cc. samples, diminishes the accuracy of the procedure, but is permissible if pH_s within ± 0.05 is sufficiently accurate.

Calculation

By means of the logarithmic chart of figure 44, CO_2 tension and pH can be determined without calculation. The two saturated serum points, A and B are marked on the chart and connected by a straight line. The carbon dioxide content of the original serum, x, is marked on this line. The CO_2 tension, P_{CO_2}, and pH can be read with an accuracy of 0.01 pH and 0.5 mm. of CO_2 tension.

The error of the graphic calculation is less than the experimental error of the method by a sufficient margin to make it doubtful whether expenditure of more labor to diminish the error of calculation is worth while. A more precise interpolation can, however, be obtained by making use of equation 5 to calculated the CO_2 tension sought.

$$\text{(5)} \qquad \log p_x = \log p_B - \frac{(\log C_B - \log C_x)\,(\log p_B - \log p_A)}{(\log C_B - \log C_A)}$$

C = CO_2 content in volumes per cent. p = tension of CO_2 in millimeters of mercury. Subscripts A and B refer to the serum or plasma samples saturated at known low and high CO_2 tensions respectively, subscript x to the sample analyzed without saturation (see figure 44).

From p_x thus obtained pH_x can be calculated by the Henderson-Hasselbalch equation 6:

$$\text{(6)} \qquad pH_x = 6.10 + \log \frac{C_x - 0.067\, p_x}{0.067\, p_x}$$

(Equation 6 is equation 14 on page 880 of volume I, with the numerical factor 0.0301 replaced here by 0.067 in order to apply the equation to CO_2 contents expressed in volumes per cent instead of millimols per liter.)

Less accurate method by determination of a single point on the separated serum CO_2 absorption curve

For the method just described, if 1-cc. samples are used for analysis at least 8 cc. of serum are required. It is, however, possible with somewhat greater error to determine P_{CO_2} and pH from the carbon dioxide content and 1 point of the absorption curve, thereby diminishing by 3 cc. the amount of serum required. Eisenman found that the average change of CO_2 content of separated serum between 30 and 60 mm. of CO_2 tension at 38° was 5.6 volumes per cent. If, then, the CO_2 content of separated serum at any known tension of CO_2 is determined, the probable absorption curve is defined by a line drawn on logarithmic paper through the observed point, in such a manner that its intersection with the 30 mm. ordinate is 5.6 volumes per cent of CO_2 lower than its intersection with the 60 mm. ordinate. On this line a point representing the CO_2 content of the true serum is placed. If the CO_2 tension of the circulating blood is reasonably near that at which the serum is saturated, the error of the method is small, but it increases as the difference between the two tensions increases.

The one-point procedure therefore consists in saturating, at a determined CO_2 tension near the probable tension of the drawn blood enough serum for one analysis. From the CO_2 tension and content of this sample the CO_2 absorption curve of the serum is plotted on the logarithmic chart as above outlined. Another sample of the serum, kept during and after centrifugation without gas change, is analyzed to obtain C_X, which is interpolated on the absorption curve. From the interpolated point the desired CO_2 tension and pH are found by inspection of the chart.

DIRECT DETERMINATION OF CO_2 TENSION AND pH IN WHOLE BLOOD AND URINE.* VAN SLYKE, SENDROY, AND LIU (63c)

Discussion

In this procedure whole blood is is equilibrated with $\frac{1}{9}$th its volume of gas which contains CO_2 and O_2 in tensions approximating those of the blood. Exchange of CO_2 between the blood and the gas bubble quickly brings the CO_2 tension in the bubble to the same level as in the blood. Because of the large amount of hemoglobin buffer in whole blood, the amount of CO_2 that the blood gives off to or takes from the gas has, under these conditions,

*The details are here described for blood. Drs. Sendroy and Seelig have shown that the same technique applies also to urine. The only change required is to equilibrate 50 cc. of urine, instead of 9 cc. of blood, with 1 cc. of gas.

negligible effect on the CO_2 tension and pH of the blood. The final CO_2 tension of the system is, within a fraction of a millimeter, the same as the initial CO_2 tension of the blood. The final CO_2 tension of the gas is determined by micro gas analysis of the bubble, by the method of Van Slyke, Sendroy, and Liu, described on pages 119–122 of chapter III.

The blood is anaerobically transferred to a centrifuge tube, and is centrifuged while protected from contact with air.

From the CO_2 content of the plasma and the CO_2 tension found by analysis of the gas bubble, the plasma pH is estimated, as described above for Eisenman's method, by Hasselbalch's equation, either algebraically by equation 6 or graphically by means of figure 41 or 44.

As an alternative to centrifugating the whole blood and determining the CO_2 content of the plasma, one may determine the CO_2 in the whole blood (either as drawn or after equilibration, since the difference is not significant). The CO_2 content of the plasma is then estimated from that of the whole blood by means of the line chart in figure 40, and the estimated plasma CO_2 content is used in calculating pH. This indirect estimation of plasma CO_2 content in place of direct analysis may introduce an error of as much as two volumes per cent of plasma CO_2, and a resultant error of 0.02 in pH_s, although the usual limit of error is only half as much. Even with indirect estimation of plasma CO_2 content from whole blood analyses, the average deviation of gasometric pH_s values from electrometric ones has been found to average not over 0.02 pH, and in no case (48 determinations) to exceed 0.04 pH (63*c*). With direct plasma CO_2 analyses the errors are about 0.01 pH less.

That, under the conditions of the equilibration used, the final CO_2 tension of the gas is brought to very nearly the initial tension of the blood may be seen from the following calculations. One cubic centimeter of gas is equilibrated with 9 cc. of blood. If we assume that the initial tension of the gas is 40 mm., while that of the blood is 50 mm. (a larger difference than will usually occur), the gas bubble, in order to raise its CO_2 tension to 50 mm., will take from the blood 0.0126 cc. of CO_2 (calculated at 0°, 760 mm.), and thereby reduce the blood CO_2 content by 0.14 volume per cent. The fall in CO_2 tension caused by removing this amount of CO_2 from normal whole blood is only 0.3 mm. (see figure 91 of volume I).

If the oxygen content of the blood changed markedly during equilibration the CO_2 tension would be affected, because of the well known influence of oxygenation in raising CO_2 tension. However, the exchange of O_2 between the gas bubble and the blood under the conditions of the equilibration has been found (63*c*) to have no significant effect on the results, even in the case of venous blood where the initial O_2 tension is the most variable.

Reagents

The reagents are those required for "Carbon dioxide in respiratory air by isolation method" described on page 119 of chapter III, and those required for determination of CO_2 in blood or plasma, described in a preceding section of this chapter.

Apparatus

For preparing the gas mixtures with which the blood is equilibrated the apparatus shown in figure 45 is used, without the gas burette or the mercury valve in the lower right corner.

For equilibrating the blood with a bubble of 1 cc. of gas the apparatus shown in figure 46 *B* is used. The chamber is of 10-cc. capacity, and the contracted portion of the top is marked to contain 1 cc. of gas over 9 cc. of blood. The stop cocks and all capillaries leading to them are of 1 mm. bore. The bulb *G* is of 1 cc. capacity. The gas is transferred to *G* after equilibration, and is kept in it over mercury until convenient time for analysis.

Preparation of gas mixtures used for equilibration

The technique is essentially the same described for "preparation of air-CO_2 mixtures by pressure" on page 301. However, the capillary which is shown in figure 45 below cock *d* to be connected with a gas burette is in this case connected with an oxygen tank, and the inlet to the water trap in the lower right corner is connected with a supply of hydrogen or nitrogen gas instead of air. The mercury trap is omitted.

A Barcroft tonometer of 100 to 200 cc. capacity is convenient for making up the gas mixture. It is attached to the manifold as shown in fig. 45.

The tonometer is evacuated 3 times and filled, after each evacuation, with H_2 or with N_2. It is then evacuated again, and the system is tested for leaks, as described on page 303. CO_2 and O_2 are then admitted until the pressure after each admission has fallen by the desired number of millimeters. Then as much H_2 or N_2 is admitted as will enter through cock *f* (fig. 45).

For venous blood 50 mm. of CO_2 tension and 25 mm. of O_2 tension are used.

For arterial blood 40 mm. of CO_2 tension and 80 mm. of O_2 tension are used.

If the CO_2 tension of the blood is believed to be abnormally high or low, CO_2 tensions greater or less than the above are used.

In each case the manometer is allowed to fall the full number of millimeters corresponding to the desired tension. For the Eisenmann method only 0.9 of the desired tension of each gas was measured at room temperature, because the equilibration was subsequently carried out at 38° in a tonometer from which none of the gas was permitted

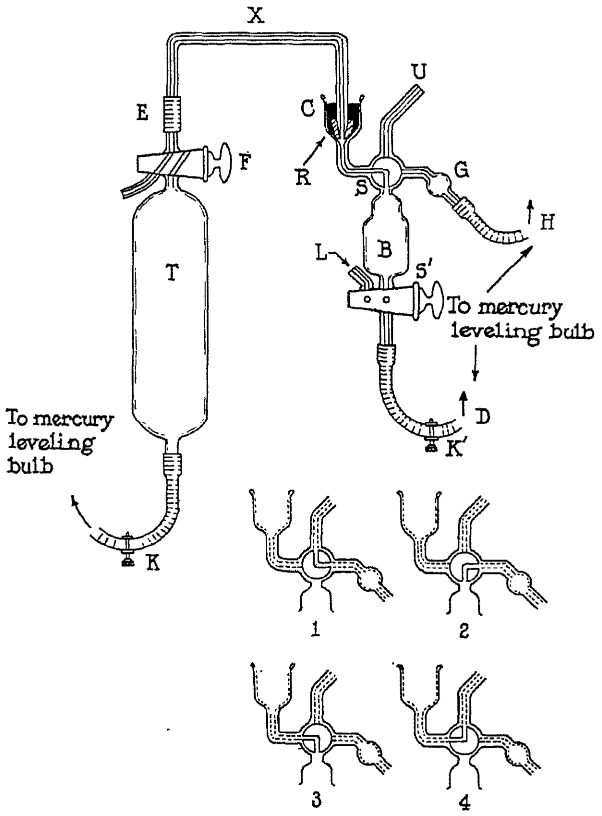


FIG. 46. Micro tonometer for equilibrating blood with 1 cc. of gas. From Van Slyke Sendroy, and Liu (63c).

to escape, so that the tension of each of the contained gases was increased about 10 per cent by the rise in temperature. In the present method, however, the pressure caused by warming the saturation vessel is released by opening a cock to the air before the equilibration is finished, so that no allowance need be made for increase in pressure when the tonometer is warmed.

It is sufficient if the mixtures are made up to within 1 or 2 mm. of the designated tensions, since the final CO_2 tension in the relatively small gas bubble is determined by the initial CO_2 tension of the blood, and not, as in the Eisenmann method, by the initial CO_2 tension of the gas.

Introduction of gas into the micro tonometer

The large container, T, in fig. 46, contains the prepared gas mixture. The chamber *B* (fig. 46) and the 3 capillary tubes at its top are filled with mercury, and the bulb *G* is filled with mercury from *H*. Cock *S* is placed in position *3* and a drop of caprylic alcohol is drawn from the cup *C* into the capillary below the cup. Cup *C* is then partially filled with mercury from above, and *B* and *T* are connected as shown in the figure. The connecting capillary *X* is of 0.5-mm. bore, and has at its tip a tapered rubber ring, shown inserted into the cup *C*. About 3 cc. of mercury are admitted into *T* from leveling bulb *A*, and capillary *X* is cleared of atmospheric air by connecting it with *T* and allowing gas from *T* to waste through *X* and bubble out through the mercury in *C*. The interiors of *B* and *T* are then connected by turning the proper cocks, and by lowering the leveling bulb *D* the mercury is withdrawn from *B* and is replaced with gas from *T*. Stop-cocks *S'*, *S*, and *F* are closed in the order given. *S* is left closed between positions *2* and *3*. Clamp *K'* is closed and the rubber tube is disconnected from *S'*. Capillary *X* and the mercury are removed from cup *C*.

Introduction of blood into the micro tonometer

The blood sample is collected anaerobically and without stasis, as described in chapter II, in a tube which contains enough potassium oxalate and ammonium fluoride to make the final concentration of these substances in the blood 0.2 and 0.1 per cent respectively. The necessary volume of a **neutral** solution containing 20 grams of potassium oxalate and 10 grams of ammonium fluoride per 100 cc. is spread as a film over the inside of the blood collecting tube, and the film is dried with a current of air. The blood is preferably collected directly over mercury (fig. 6) but may be collected under oil and thence transferred to a tube over mercury.

To the tube containing the blood over mercury the gas-filled micro tonometer *B* (fig. 46) is connected by means of a glass capillary from the upper outlet of the blood tube to the inlet below cock *S'* of the micro tonometer. Air and the first few drops of blood from the blood

tube are wasted through outlet *L* in cock *S'*. Cock *S* is then turned to position *3*, and blood is admitted to the tonometer until the fluid reaches the mark, and the chamber contains 9 cc. of blood and 1 cc. of gas. Cock *S* is then closed in position midway between *3* and *4*. Leveling bulb *d* is reconnected to the tonometer's lower inlet capillary, and that capillary is cleared of blood by passing mercury through it and the outlet *L*.

Equilibration of blood and gas at 38°

The tonometer *B* in an upright position is immersed as far as the upper cock in water at 38° ±0.1°. Leveling bulbs *D* and *H*, attached to the tonometer, are suspended outside the bath. A droplet of mercury is placed in cup *C*. The tonometer is held in the bath for one or two minutes, then *S* is turned to position *3* to allow escape of enough of the warmed air to lower the pressure within the chamber to atmospheric. The escape of gas is indicated by movement of the droplet of mercury in cup *C*. Cock *S* is closed and the vessel is left in the bath for another minute, after which *S* is again opened. This procedure is repeated until there is no further indication of the escape of gas when the cock is opened.

Cock *S* is turned from position *3* in a clockwise direction to a position midway between *1* and *2*. A rubber stopper is inserted into the mouth of cup *C* to keep out water from the bath. The entire tonometer is then immersed in the bath and is rocked in such a manner that the bubble moves from one end of the chamber to the other. Ten minutes suffice for attainment of CO_2 equilibrium. At the end of that period the vessel, still in the bath, is placed in an upright position for one or two minutes to permit drainage of blood from the wall of the upper part of the chamber. Then, with leveling bulb *H* slightly elevated, stopcock *S* is turned to position *2* just long enough to permit a droplet of mercury from G to pass into the chamber *B*. The mercury removes blood from the bore of cock *S*, from which it might otherwise enter G when the gas is transferred to this bulb.

Separation of equilibrated blood and gas

This operation is preferably performed without removing the tonometer from the bath. If one works quickly, however, the tonometer may be taken out and the gas bubble transferred to bulb *G* before temperature change has significantly affected the distribution of CO_2 between the gas and blood.

The tonometer is either removed from the bath, or, preferably, placed in an upright position with only the part above cock *S* above the surface of the bath. The stopper is removed from *C*. Leveling bulb *H* is placed slightly below and leveling bulb *D* slightly above the tonometer. Cock *S* is then turned to position *2*. A portion of the gas from *B* escapes at once into *G*. Most of the remaining gas is driven into *G* by admitting mercury from leveling bulb *D* into the bottom of chamber *B*. The admission of mercury is stopped when almost all of the gas has been transferred to *G*, and before any blood has entered the bore of cock *S*. Cock *S* is then turned to position *3* and the small bubble of gas left in *B*, followed by a little blood, is allowed to escape into cup *C*.

S is turned to position *4*, and cup *C* and the bore of the cock are cleaned by drawing water, and then acetone in succession through *U*. The separated gas and blood may now be analyzed at the operator's convenience. If, however, the blood is not analyzed at once, it should be chilled in ice water and kept cold until used. Even then the blood analysis should be made on the same day. Before removal of either the blood or gas for analysis, the tonometer should be brought to room temperature.

Determination of the CO_2 content of the gas phase

This analysis is carried out as described in chapter III under the heading, "Carbon dioxide in respiratory air by the isolation method." The technique described for measuring micro gas samples on page 121 is followed. To transfer the gas sample from bulb *G* of figure 46, the arm *U* is connected glass to glass with the side arm of the Van Slyke-Neill manometric chamber. Mercury is then run back and forth between cup *C* of the tonometer and the Van Slyke-Neill chamber to drive all gas bubbles out of the connections. Manometer reading p_0 is taken, with the meniscus of the mercury at the 2-cc. mark in the gas-free manometric chamber. The mercury leveling bulb attached to *H* is then placed higher than the leveling bulb of the manometric apparatus, cock *S* is turned to position *1*, and all the gas in *G* is passed into the manometric chamber followed by a little mercury to seal the cock of the chamber. The mercury meniscus in the chamber is again brought to the 2-cc. mark and manometer reading p_1 is taken. The pressure exerted by the gas sample at 2-cc. volume is calculated as

$$P_S = p_1 - p_0$$

The absorption of the CO_2 with NaOH solution and the rest of the analysis are then carried out as described on pages 121 to 125 of chapter III.

Centrifugation of blood and determination of plasma CO_2 content

To outlet U of the micro-tonometer B (fig. 46) is connected, glass to glass, a capillary tube so bent that its opposite end can be passed to the bottom of a centrifuge tube. The blood is then passed into a centrifuge tube containing a layer of oil. The oil is replaced by a layer of low melting paraffin, as described on page 58 of chapter II, and the blood is centrifuged. A hole is then made through the paraffin with a warm cork borer, and 1-cc. samples of the plasma are withdrawn into pipettes and used for determination of the CO_2 content as described on page 283 of this chapter.

In place of the paraffin plug to prevent escape of CO_2 during the centrifugation, one may use centrifuge tubes arranged as indicated in figure 7, II or III (p. 56).

Calculation

The CO_2 content C of the gas bubble is calculated as described for this analysis in chapter III page 122.

From the volume per cent of CO_2 in the gas phase, indicated as C, the CO_2 tension, p, is calculated by the usual formula:

$$p = 0.01\ C(B - 49)$$

where B is the barometric pressure in millimeters of mercury and 49 is the vapor tension of water at the 38° temperature of the equilibration, and, assumed, of the blood while in the circulation.

The calculation, algebraically or graphically, of the plasma pH from the CO_2 tension of the blood and the CO_2 content of the plasma, has already been explained in the third and fourth paragraphs of the introductory discussion of this determination.

CALCULATION OF PLASMA pH FROM PLASMA CO_2 CONTENT AND ALVEOLAR CO_2 TENSION

The value of the CO_2 tension in the Henderson-Hasselbalch equation 6 (p. 308) may be ascertained by determining the CO_2 tension of the alveolar air under conditions such that either the arterial or venous tensions will be obtained. Procedures for these determinations have been described in chapter IV. Arterial CO_2 tensions obtained by analysis of alveolar air have

been found by Hastings, Neill, Morgan, and Binger (16b) to agree with those found in the blood even in severe pneumonia. While one can not predict that in every pathological condition the "arterial" CO_2 tension found in the alveolar air will agree with that actually found in the arterial blood it seems probable that such exceptions even in disease are rare. It appears that as a rule arterial pH_s values calculated by figure 41 or 44 or by equation 6 given above for Eisenmann's method, with alvolar CO_2 tension in place of p_x are as accurate as the analytical determinations of serum CO_2 content and alveolar CO_2 tension.

In the case of venous blood an equal degree of accuracy can not be expected. It is clinically impossible to obtain mixed blood from the right heart, and blood from an arm vein may deviate markedly in its CO_2 content from the mixed venous blood. Also the technique of determining the venous CO_2 tension in the alveolar air is more complicated and liable to error than the technique for determinng arterial CO_2 tension in the alveolar air. When venous serum CO_2 contents must be used for calculation of pH_s values, the most convenient approximation, and as accurate as any other, is obtained by estimating the arterial serum CO_2 content from the venous, and using this value together with the arterial CO_2 tension determined in the alveolar air, for the calculation of arterial pH_s by Hasselbalch's equation. If the oxygen unsaturation of the venous blood is known the CO_2 content of the arterial serum can be estimated within 1 or 2 volumes per cent by subtracting from the venous serum CO_2 content 0.8 volume per cent for each volume per cent of oxygen unsaturation. In the lack of oxygen data one can assume that the arterial serum CO_2 content in the resting indivdual is 5 volumes per cent less than the venous and seldom be so far off as to make an error from this source alone as great as 0.05 in the calculated arterial pH_s.

However, since arterial blood can be readily obtained by skin puncture, as in the method of Shock and Hastings described in chapter 27, it should seldom be necessary to base estimates of arterial CO_2 content on analyses of venous blood.

The calculation of pH_s from serum CO_2 content and alveolar CO_2 tension can be made either algebraically by equation 6 in the preceding section on Eisenman's method, or graphically by the line chart of figure 41 or 44. The graphic method is simpler, and gives results within 0.01 pH.

CO_2 CAPACITY AND CO_2 ABSORPTION CURVE OF WHOLE BLOOD

The *CO_2 capacity* of whole blood is usually designated as the CO_2 content found after saturating the blood at 38° with air containing CO_2 at 40-mm. tension. The same technique for saturation of serum described in the pre-

ceding section on gasometric serum pH determination may be used. When whole blood is saturated it requires more time than serum to reach equilibrium, and fifteen to thirty minutes saturation are used. The larger the ratio of volume of blood to surface of tonometer, and the thicker the layer of blood on the tonometer walls during saturation, the longer will be the time required to reach equilibrium. It is desirable to have the tonometer size 50-fold the volume of the blood put in it. Then fifteen minutes suffice for equilibration, and the volume of CO_2 which the blood absorbs from, or gives off to, the gas phase with 40-mm. CO_2 tension, is ordinarily negligible. During the saturation at 38° appreciable amounts of lactic acid may form from glycolysis and cause a fall in the CO_2 capacity unless preventive measures are taken. To prevent lactic acid formation it is desirable to add 10 mg. of neutral sodium or ammonium fluoride per cubic centimeter of blood (see chapter II). The sodium fluoride greatly retards glycolysis, but the ammonium salt prevents it altogether (Stadie, personal communication).

For *CO_2 absorption curves on whole blood* one may again use the same technique described for serum. However, because of its greater buffer content, the blood when saturated with CO_2 at tensions above or below that in the circulation, takes up or gives off much larger amounts of CO_2 than does serum, when equilibrated at high and low levels of CO_2 tension. Therefore, if one uses the technique previously described one is routinely compelled to correct, by means of equation 2 on page 304, for CO_2 given off or absorbed by the blood during equilibration.

There are two means of avoiding such correction. One is to transfer the saturated blood to a fresh tonometer, and saturate a second time with gas mixture of the initial composition. The transfer may be accomplished by connecting the bottom of the blood-containing tonometer (T in figure 45) by means of a U-shaped capillary tube with the fresh tonometer, which has already been charged with the proper amount of CO_2. A partial vacuum is left in the fresh tonometer, so that when the connections are opened the blood is drawn over. It may be necessary to resaturate in a third tonometer before the CO_2 content of the whole blood becomes constant at a CO_2 tension widely divergent from the original. The other way to avoid correction for CO_2 exchange between blood and gas is to use the double tonometer method described below.

For saturation curves of whole blood the most accurate and convenient procedure in the authors' experience is the use of the *double tonometer* (fig. 47) described by Austin *et al* (3) as their "first saturation method." The smaller chamber is from $\frac{1}{20}$th to $\frac{1}{50}$th the volume of the larger, and is of capacity to contain somewhat more than the volume of blood to be saturated. The two

chambers are connected by a rubber tube of 6 or 7 mm. bore. With this technique one does not attempt to fix exactly the final CO_2 tension when the gas mixture is made up. The desired tension is approximated at the start, and the actual tension at equilibrium is determined by analysis of the gas phase, the gas and blood after equilibration being separated for analysis in the two chambers of the tonometer.

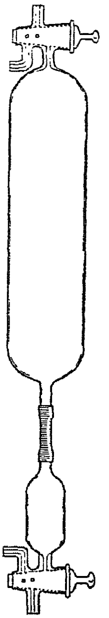


FIG. 47. Double tonometer for saturating blood with gas mixtures of analyzed composition. From Austin et al. (3).

Before equilibration the blood is placed in the smaller chamber, the two chambers are connected with the rubber tube, and the tube is closed by a screw clamp. The tonometer is then connected to a gas manifold like the one shown in figure 45 (gas burette is unnecessary in this case). The tonometer is more or less completely evacuated, and the pressure on the manometer is read. The pump is shut off from the tonometer, and the manometer, still connected with the tonometer, is observed for a minute or two in order to see whether a leak is indicated by falling of the mercury. If the apparatus proves to be tight the manometer is read, and then CO_2 is admitted into the system

until the mercury in the manometer has fallen 30, 40, 60, or whatever number of millimeters corresponds to the desired CO_2 tension. Air or other gas is then admitted to bring the total pressure in the tonometer up to atmospheric (the mercury valve shown in the lower right corner of figure 45 is omitted in this technique). By using a manifold with additional connections to sources of H_2, N_2, and O_2, any desired mixture with these gases can be made. The use of the manometer to measure the amounts put in obviates the necessity of knowing the size of the tonometer, and of calculating the volume of CO_2 to add.

For saturation nearly all of the blood is run into the larger chamber of the tonometer, and the tonometer is rotated in a horizontal position in a bath at the desired temperature, usually 38°. From time to time one cock is brought outside the bath and opened. This is repeated until the absence of positive pressure in the tonometer shows that it has reached thermal equilibrium with the bath. (The drop of water at the mouth of the capillary is no longer pushed away by internal pressure when the cock is brought outside the bath and opened.) The rotation of the tonometer is then continued for fifteen to twenty minutes.

At the end of the equilibration period the tonometer is placed upright in the bath, with the smaller chamber below the larger, so that the blood drains into the smaller. When drainage is as complete as possible, two clamps are screwed onto the rubber tube between the chambers (fig. 47) and the tube is cut in two between the clamps.

The larger chamber is now removed from the bath, and the gas in it is analyzed for CO_2, and for other gases if desired, at leisure. From the analysis the tension of CO_2 or of other gases, at the end of the equilibration is calculated by the usual formula:

$$p = \frac{C}{100} \times (B - W)$$

where p is the tension, C the per cent of CO_2 or other gas found in the gas mixture, W is the vapor tension, 49 mm. at 38°.

After the larger chamber is removed, the three-way cock of the smaller chamber is brought above the surface of the bath, is connected with a mercury leveling bulb, and a little mercury is wasted through the three-way cock to remove air and water from its bore. The chamber is then inverted and fastened in position with the cut end of the clamped rubber tube projecting from the bath. Water is removed from the bore of the tube above the clamp. Then the clamp is opened, and mercury is admitted into the bottom of the chamber until the gas is

displaced and blood rises into the rubber tube. This is then closed again with the clamp. The chamber now serves as a container for preserving the blood over mercury, as described in connection with figure 6 on page 54 of chapter II. Samples of blood for analysis are drawn from the chamber as described under "Measuring blood samples" in chapter II.

OXYGEN IN BLOOD. VAN SLYKE AND NEILL (59)

With the same manometric apparatus oxygen determinations can be performed on blood samples varying from 2 to 0.1 cc. in size. With samples of 2, 1, or 0.5 cc. one can, with some experience, obtain duplicate results differing by an average of less than 0.05 volume per cent of oxygen. Such results are, of course, obtained with less attention to detail when the larger samples are used. But when one has become familiar with the apparatus it is practically as easy to obtain them with 0.5 cc. as with 2-cc. samples. It is necessary that the 0.5-cc. sample be smoothly delivered from a pipette calibrated to within 1 cubic millimeter, and that when the manometer readings are made a lens shall be used to locate the meniscus of the blood solution exactly on the 0.5-cc. mark in the chamber of the apparatus, and to locate the mercury meniscus in the manometer. For the necessary accuracy in locating the meniscus of the blood solution a frosted light placed behind the chamber to reveal the meniscus sharply is required. With the aid of such a light and a reading lens, however, one has no difficulty in obtaining highly accurate results with 0.5-cc. blood samples. In a considerable series of analyses by one of the writers the average difference between duplicates was 0.03 volume per cent of O_2, corresponding to an average difference in duplicate manometer readings of 0.25 mm.

With samples smaller than 0.5 cc. the accuracy diminishes in proportion to the size of the sample, but samples of 0.2 cc. can yield results with mean error not exceeding 0.2 volume per cent of oxygen.

Reagents

1 N NaOH, neutral ferricyanide-saponin solution, and 20 per cent hyposulfite described previously under "General reagents." The NaOH and hyposulfite are prepared air-free as described on p. 235.

Procedure

For 2 cc. of blood, 10 cc. of the neutral ferricyanide-saponin reagent mixture are placed in the chamber and freed of air by three minutes extraction.

In freeing the reagent solution from air it is not possible in the chamber of the manometric apparatus to separate entirely the mercury from the solution. The contact is kept sufficiently small to prevent significant reaction with the ferricyanide, however, if the solution is drawn so far down into the tube at the bottom of the chamber that ***none of the mercury is thrown up and rotated about the bulb during the extraction of the ferricyanide reagent.***

The extracted air is ejected from the chamber and 6 cc. of the ferricyanide solution are forced up into the cup above the chamber. It is advisable before this moment to have the blood sample ready in a pipette. As quickly as possible after the 6 cc. of reagent solution have been forced up into the cup, the blood sample is run into the chamber of the apparatus, as described in connection with either figure 29 or 30. One cubic centimeter of the reagent solution is run into the chamber after the blood, leaving 5 cc. in the cup. This portion left in the cup is now discarded. Its purpose has been to serve as a protecting layer to prevent diffusion of atmospheric air into the 1 cc. at the bottom, which is used to wash the last drops of blood into the chamber. Some water is placed temporarily in the cup, and the cock is sealed by filling its bore with mercury.

The chamber is now evacuated, and the oxygen is extracted from the solution by shaking 3 minutes.

When extraction is complete mercury is admitted from the leveling bulb into the chamber until the gas volume has been reduced to about 5 cc., and the CO_2 is absorbed with air-free 1*N* sodium hydroxide, as described on p. 284 for determination of CO_2 in whole blood.

After one minute has been allowed for the alkali to finish draining down the walls of the upper part of the chamber, the fluid meniscus in the chamber is brought to the 2 cc. mark and reading p_1 is taken on the manometer, the gases in the chamber being the oxygen and nitrogen extracted from the blood. A frosted light behind the chamber should be used to outline sharply the meniscus of the dark blood solution.

To absorb the O_2 the chamber is evacuated until a gas space of 4 or 5 cc. is obtained. The cock leading to the mercury bulb is then closed. 1.5 cc. of air-free solution of hyposulfite and anthrahydroquinone betasulfonate are placed in the cup of the apparatus, and part of the solution is admitted to the chamber, a drop at a time. As each drop trickles down the inner wall of the chamber it absorbs oxygen, and the mercury in the manomater falls. After a few drops have been admitted no further perceptible fall occurs. The cock leading to the mercury leveling bulb

is then opened and the solution in the chamber is permitted to rise as near to the stopcock as it will, with the mercury leveling bulb in the position shown in figures 36 and 37. The remainder of 1 cc. of hyposulfite solution is then added in several portions, completing the absorption of the last traces of oxygen. The entire absorption can thus be completed in about one minute.

After absorption of O_2 is completed the p_2 reading is taken with the gas volume again at 2 cc. The oxygen pressure is

$$P_{O_2} = p_1 - p_2 - c.$$

The value c is determined by extracting for three minutes 10 cc. of the ferricyanide reagent solution. Three cubic centimeters are then ejected and the remaining 7 cc. in the chamber are extracted two minutes. At the end of the extraction 1 cc. of air-free sodium hydroxide is admitted from the cup. The manometer is read with the gas volume at 2 cc., and 1 cc. of hyposulfite is run in. The reading is then repeated. The difference between the two readings is the correction c. The c value thus determined combines the correction for the effect of 1 cc. of hyposulfite on the level of the mercury in the chamber with a slight correction for dissolved atmospheric oxygen not removed from the ferricyanide solution by one preliminary extraction.

For ***oxygen determination in 1 cc. of blood*** the technique is the same, except that less ferricyanide solution is used. 7.5 cc. of the ferricyanide solution are first freed of air by three minutes shaking, 6 cc. are run up into the cup, and 1 cc. is returned with the blood sample to the chamber, so that the final mixture in the latter is 1 cc. of blood with 2.5 cc. of the ferricyanide solution.

For ***oxygen determination in 0.5 cc. or less of blood*** 6 cc. of the ferricyanide solution and a drop of caprylic alcohol are freed of air by two minutes extraction in the chamber, and 5 cc. of the solution are run up into the cup. The blood sample is then run into the chamber from a simple bulb pipette calibrated for complete delivery. The technique of delivery is that described on page 241 for "Delivery from rubber tipped pipette without stop-cock." After the blood has been delivered enough ferricyanide from the cup is admitted to the chamber to bring the total volume of fluid there to 2 cc.

As an alternative to the mode of delivery directed above, 0.1 or 0.2 samples of blood may be delivered from pipettes calibrated to deliver between two marks, and not provided with rubber rings. The blood sample is deposited on the floor of the cup underneath the ferricyanide

solution, and is then admitted to the chamber followed by enough ferricyanide solution to bring the fluid volume in the chamber to 2 cc. A few cells are likely to remain adherent to the floor of the cup, but the loss in accuracy appears to be not important for the micro analyses. For the accurate analyses with 0.5 cc. samples the delivery prescribed in the preceding paragraph is desirable.

The rest of the analysis is carried out in the same manner described for 2-cc. samples, with one exception in the case of 0.2 or 0.1-cc. samples. When in these micro analyses the oxygen is absorbed with hyposulfite, the preliminary admission or several drops into the partially evacuated chamber is omitted. After the p_1 reading is taken the pressure in the chamber is allowed to rise to nearly atmospheric, and the hyposulfite (of which 0.5 cc. suffices) is admitted in several portions over the small bubble of gas that lies under the cock at the top of the chamber.

To determine the c correction 6 cc. of ferricyanide solution and a drop of caprylic alcohol are freed of air by two minutes extraction in the evacuated chamber. Four cubic centimeters are forced up into the cup, and the remaining 2 cc. are extracted, and the p_1 and p_2 readings are taken after admission of alkali and hyposulfite respectively, as in the blood analysis. The value of the p_1–p_2 difference obtained in this blank analysis constitutes the c correction.

Calculation of blood oxygen content

The oxygen pressure is calculated as

$$P_{O_2} = p_1 - p_2 - c$$

The P_{O_2} thus found is multiplied by the proper factor from table 30 to calculate the oxygen content of the blood in millimols per liter or in volumes per cent.

SIMULTANEOUS DETERMINATION OF CARBON DIOXIDE AND OXYGEN IN 1 CC. OF BLOOD. VAN SLYKE AND NEILL (59)

The procedure is the same as that for determination of oxygen in 1 cc. of blood, except that instead of the neutral ferricyanide solution the acidified solution is used, and the pressure reading is taken before as well as after absorption of the carbon dioxide with 1 N sodium hydroxide.

TABLE 30

FACTORS FOR CALCULATION OF O_2, CO OR N_2 CONTENT OF BLOOD (FROM VAN SLYKE AND NEILL (59))

TEMPERATURE	FACTORS BY WHICH MILLIMETERS P_{O_2}, P_{CO}, or P_{N_2} ARE MULTIPLIED TO GIVE											
	Millimoles O_2, CO or N_2 per liter of blood						Volume per cent O_2, CO or N_2 in blood					
	Sample = 0.2 cc. S = 2.0 cc. a = 0.5 cc. i = 1.00	Sample = 0.5 cc. S = 2.0 cc. a = 0.5 cc. i = 1.00	Sample = 1 cc. S = 3.5 cc.		Sample = 2 cc. S = 7 cc.		Sample = 0.2 cc. S = 2.0 cc. a = 0.5 cc. i = 1.00	Sample = 0.5 cc. S = 2.0 cc. a = 0.5 cc. i = 1.00	Sample = 1 cc. S = 3.5 cc.		Sample = 2 cc. S = 7 cc.	
			a = 0.5 cc. i = 1.00	a = 2.0 cc. i = 1.00	a = 0.5 cc. i = 1.00	a = 2.0 cc. i = 1.00			a = 0.5 cc. i = 1.00	a = 2.0 cc. i = 1.00	a = 0.5 cc. i = 1.00	a = 2.0 cc. i = 1.00
°C.												
15	0.1389	0.05556	0.02780	0.1113	0.01396	0.0558	0.312	0.1246	0.0623	0.2493	0.0317	0.1251
16	84	38	70	09	90	56	10	42	21	85	15	46
17	80	20	61	05	85	54	09	37	19	78	14	42
18	75	00	51	01	80	52	08	33	17	68	12	37
19	70	0.05480	41	0.1097	75	50	07	29	15	59	11	32
20	65	60	31	93	70	48	07	24	13	50	09	28
21	60	40	21	89	65	46	06	20	10	41	08	24
22	55	20	11	85	60	44	05	16	08	32	06	19
23	50	00	02	81	55	42	03	11	06	23	05	15
24	45	0.05380	0.02692	77	50	40	02	07	04	14	03	10
25	40	60	83	74	45	38	01	0.1203	02	06	02	06
26	35	40	73	70	41	36	00	0.1199	00	0.2398	01	02
27	31	22	64	67	36	34	0.299	95	0.0598	90	0.0299	0.1198
28	26	04	55	63	31	32	98	91	96	82	98	93
29	22	0.05286	47	59	27	30	97	87	93	74	96	89
30	18	70	38	55	22	29	96	83	92	66	95	85
31	13	52	29	52	18	27	95	79	90	58	94	81
32	09	34	20	48	14	25	94	75	88	50	92	77
33	04	16	11	44	09	24	93	71	86	42	91	73
34	00	00	02	41	05	22	92	67	83	33	90	69

In Table 31 are given the corrections for dissolved gases to be subtracted when combined O_2 is to be estimated from total O_2.

TABLE 31

ESTIMATED CORRECTIONS FOR DISSOLVED O_2 AND N_2 IN BLOOD (FROM VAN SLYKE AND NEILL (59))

BLOOD	DETERMINED	SOUGHT	CORRECTION TO SUBTRACT	
			vol. per cent	*mM per l.*
Venous	Total O_2	Combined O_2	0.1 (O_2)	0.04 (O_2)
Arterial	Total O_2	Combined O_2	0.2 (O_2)	0.09 (O_2)
Saturated with air at 20°, 760 mm.	Total O_2	Combined O_2	0.5 (O_2)	0.22 (O_2)
Venous	Total $O_2 + N_2$	Combined O_2	1.3 ($O_2 + N_2$)	0.57 ($O_2 + N_2$)
Arterial	Total $O_2 + N_2$	Combined O_2	1.4 ($O_2 + N_2$)	0.62 ($O_2 + N_2$)
Saturated with air at 20°, 760 mm.	Total $O_2 + N_2$	Combined O_2	1.9 ($O_2 + N_2$)	0.85 ($O_2 + N_2$)
Saturated with air at 20° 760 mm.	Total $O_2 + N_2$	Total O_2	1.4 (N_2)	0.62 (N_2)
Venous	Total $O_2 + N_2$ or $CO + O_2 + N_2$	Total O_2 or $CO + O_2$	1.2 (N_2)	0.53 (N_2)
Arterial	Total $O_2 + N_2$ or $CO + O_2 + N_2$	Total O_2 or $CO + O_2$	1.2 (N_2)	0.53 (N_2)

Reagents

Acid saponin-ferricyanide solution.
1 N sodium hydroxide solution, air-free.
Hyposulfite solution, air-free.
Caprylic alcohol.
These are described under "General reagents."

Procedure

The ferricyanide solution is extracted air-free in the chamber and the blood sample is introduced as described above for oxygen determination.

When the blood is run down into the acid ferricyanide solution in the chamber of the apparatus a precipitate forms from a combination of the blood proteins with the reagents. As soon as the cock of the chamber has been sealed with a drop of mercury the leveling bulb of the apparatus is raised and lowered a few times, so that the solution and precipitate in the top of the chamber are drawn down into the enlarged lower part and then permitted to rise back to the cock again. This procedure detaches the precipitate from the wall and breaks it up into fine granules which remain suspended in the solution and do not interfere with any subsequent steps in the analysis.

The gases are extracted by shaking for 3 minutes. The gas volume is then reduced to 2 cc. with the precautions described for CO_2 determinations under "Adjustment of gas volume," on p. 277, and the pressure p_1 is read.

Absorption of the carbon dioxide with air-free 1 N hydroxide is carried out as described above for the oxygen determinations. As the alkali mixes with the blood solution the precipitate mentioned above dissolves. The mixing of the blood and alkali is completed by moving the chamber back and forth slightly two or three times with the hand. Vigorous shaking of the alkaline solution is to be avoided, or reabsorption of oxygen to a slight but measurable extent may occur. Finally, the gas volume is restored to 2 cc., and the pressure p_2 is read.

$$P_{CO_2} = p_1 - p_2 - c_{CO_2}.$$

After the p_2 reading the oxygen is absorbed with 1.0 cc. of hyposulfite solution, as already described for oxygen determinations, and p_3 is read on the manometer with the gas volume in the chamber at 2 cc.

$$P_{O_2} = p_2 - p_3 - c_{O_2}.$$

The nitrogen content also may be determined. A p_4 reading is taken with the gas at 0.5 cc. volume, the residual gas is ejected, the pressure in the gas free chamber is reduced again till the solution meniscus is on the 0.5-cc. mark and p_5 is read.

$$P_{N_2} = p_4 - p_5 - c_{N_2}.$$

The c corrections are determined by extracting 7.5 cc. of 0.1 N sodium hydroxide for three minutes, ejecting 4 cc. of the solution and carrying out the pressure readings and the addition of the hydroxide and hyposulfite solutions as above described. The c_{N_2} is a slight correction for a trace of nitrogen not removed in the preliminary extraction of the reagents.

The results are calculated by tables 28 (p. 280) and 30 (p. 325).

CARBON MONOXIDE IN BLOOD, WITH OR WITHOUT OXYGEN AND CARBON DIOXIDE. VAN SLYKE AND NEILL (59)

This procedure is carried out with the usual blood gas technique, and yields values for the CO content that are accurate within 0.2 volume per cent. It is therefore adequate for detection of carbon monoxide poisoning, in which several volumes per cent of CO are present. When, for special purposes, greater accuracy is desired, the more elaborate method of Sendroy and Liu, described below, may be used. The ferricyanide solution must be acidified in order to assist in overcoming the affinity of CO for hemoglobin.

Reagents

Acid ferricyanide-saponin solution.
1 N sodium hydroxide solution, air-free.
Hyposulfite solution, air-free.
Caprylic alcohol.
These are described under "General reagents."

Procedure

Of the acid ferricyanide solution 10 cc. are extracted free of air and 6 cc. are run up into the cup of the manometric apparatus as in oxygen determinations. Two cubic centimeters of blood are measured into the chamber, followed by 1 cc. of reagent solution from the cup, as in oxygen determination. The blood, with 5 cc. of the air-free reagent, is extracted for three minutes in the evacuated chamber. Carbon dioxide, carbon monoxide, and oxygen are liberated together.

The CO_2 is absorbed with 1 cc. of air-free 1 N NaOH, as described above for oxygen determinations, and reading p_1 is taken, with the gases, O_2, CO, and N_2 in the chamber at 2-cc. volume.

The oxygen is then absorbed with 1 cc. of hyposulfite.

At this point, caution is required because of the high affinity between CO and reduced hemoglobin, which can recombine rapidly with CO to form HbCO. The formation of reduced hemoglobin occurs as soon as the hyposulfite mixes with the blood solution, and can be seen by the change from the dark methemoglobin color to the clear red of reduced hemoglobin. As soon as the reduced hemoglobin has been formed in the solution the latter begins to reabsorb the CO that has been extracted from it. If unnecessary agitation is avoided, however, the proportion reabsorbed was found by Van Slyke and Neill (59) under the conditions of the analysis to be approximately constant, 2.4 per cent of the total CO present. (Since this factor is empirical it is well for the analyst to redetermine it with his own apparatus and technique, by analyzing in the same way some blood from which the oxygen has been completely displaced by carbon monoxide. The percentage of the total CO absorbed when hyposulfite is run in, as described below, is used as a correction instead of 2.4 if it is found to be different.)

The hyposulfite is run into the chamber, in the manner above described for O_2 determination, without agitation of the solution. As soon as the absorption of O_2 is complete, the cock leading to the leveling bulb is closed and the leveling bulb is placed in the lowest position. The level of the fluid in the chamber is then quietly lowered to the 2-cc. mark. Care is taken not to overrun the mark, as the manipulation of the solution to bring it back would increase the amount of CO absorbed. The liquid level in the chamber should be lowered at a sufficiently slow rate also to permit complete drainage of the solution down the walls.

The pressure p_2 is read, with the gases CO and N_2 in the chamber at 2 cc. volume.

The gas is then ejected from the chamber and pressure p_3 is read.

The c corrections are determined as usual in blank analyses, c_{O_2} being found as $p_1 - p_2$ and c_{CO+N_2} as $p_2 - p_3$.

Calculation

The pressures of the respective gases are calculated as follows:

$$P_O = p_1 - p_2 - c_{O_2} - 0.024\,(p_2 - p_3)$$

$$P_{CO+N_2} = 1.024\,(p_2 - p_3 - c_{CO+N_2}).$$

The O_2 content and the $CO + N_2$ content of the blood are calculated from P_{O_2} and $P_{CO_2+N_2}$ by the factors in table 30. From the content of $CO + N_2$ one subtracts 1.2 volume per cent for the N_2 to obtain the CO.

$$\text{Volume per cent CO} = \text{Volume per cent } (CO + N_2) - 1.2$$

If one wishes ***to determine only carbon monoxide*** without oxygen, the addition of NaOH can be omitted, since the alkaline hyposulfite will absorb both CO_2 and O_2 The readings p_2 and p_3 can then be taken with the gases at either 2.0 or 0.5-cc. volume, according to whether much or little CO is present.

The analysis can also be carried through, with somewhat less accuracy, on 1 cc. of blood. In this case CO_2 determination, as well as O_2 and CO can be included, by making a reading with the extracted gases at 2-cc. volume before the CO_2 is absorbed with NaOH.

CARBON MONOXIDE IN BLOOD, WITH OR WITHOUT OXYGEN AND CARBON DIOXIDE. PRECISE METHOD OF SENDROY AND LIU (32)

Discussion

The above Van Slyke-Neill procedure for carbon monoxide determination is simple and sufficiently exact for most analyses. It is, however, not so exact as an oxygen determination for two reasons:

1. An empirical correction must be used for the small but measurable reabsorption of CO by reduced hemoglobin that occurs during the interval between addition of hyposulfite and measurement of P_{CO+N_2}.

2. The CO can not be determined directly by absorption with cuprous chloride solution, because introduction of the latter into the chamber with the alkaline blood solution would cause an unmanageable precipitate. To make possible the use of cuprous chloride it is necessary first to remove the alkaline blood mixture from the chamber. This was accomplished by Harington and Van Slyke (16) by elaborating the extraction chamber through adding to its lower end a three-way cock and bulb, by means of which solutions could be removed from the chamber while the gases were left in it. The disadvantage was that it required replacement of the usual extraction chamber by a special one not required for any other analysis.

This inconvenience has been overcome by Sendroy and Liu (32). After extracting the blood gases in the usual Van Slyke-Neill chamber they remove them to the modified Hempel pipette (figure 48) introduced into the manometric technique by Van Slyke and Hiller (56). In the pipette the O_2 and CO_2 are absorbed by alkaline pyrogallol solution. The blood mixture

in the extraction chamber is replaced by water. The CO and N_2 from the Hempel pipette are then returned to the chamber, and the CO is measured by absorption with cuprous chloride. The carbon monoxide is thus determined directly, and with an accuracy equal to that of oxygen determinations.

The attainable accuracy is proportional to the size of blood sample used. With 5-cc. samples the error is 0.01 to 0.02 volume per cent of CO; with 1-cc. samples it is 0.05 to 0.10 volume per cent.

TABLE 32

FACTORS BY WHICH P_{CO} IS MULTIPLIED TO CALCULATE VOLUME PER CENT OF CO WHEN 5 CC. OF BLOOD ARE ANALYZED (VAN SLYKE AND ROBSCHEIT-ROBBINS (60))

TEMPERATURE	FACTOR WHEN P_{CO} IS MEASURED WITH GAS AT 0.5-CC. VOLUME	FACTOR WHEN P_{CO} IS MEASURED WITH GAS AT 2.0-CC. VOLUME
°C.		
15	0.01263	0.0505
16	58	03
17	53	01
18	49	0.0499
19	44	98
20	39	96
21	35	94
22	30	92
23	25	90
24	21	88
25	16	86
26	12	85
27	07	83
28	03	81
29	0.01199	80
30	95	78
31	90	76
32	86	74
33	82	73
34	78	71

Before using this method one should read the section on "Use of the modified Hempel pipette" on page 280.

Reagents

Acid saponin-ferricyanide solution, air-free 1 N NaOH, and air-free *Winkler's cuprous chloride solution*, described under "General reagents for blood gas determinations."

Alkaline pyrogallate solution described for use in the Haldane air analysis apparatus in chapter III, page 96. The pyrogallate is placed in the modified Hempel pipette and protected from air by a layer of paraffin oil (fig. 48).

Precise determination of small amounts of CO in blood, with 5-cc. blood samples

The procedure is a modification of that developed by Van Slyke and Robscheit-Robbins (60) to determine 1 or 2 volumes per cent of CO with an error of only 0.01 volume per cent. The primary purpose was to make possible determination of the volume of circulating blood in an animal by the carbon monoxide method of Gréhant and Quinquad (12) without saturating, as had previously been necessary, as much as one-third of the blood hemoglobin with CO in order to obtain accurate results. A technique requiring the saturation of only one-tenth or one-twentieth of the circulating hemoglobin makes the method more safe and convenient for use with human subjects, and with animals permits the introduction of the CO by intravenous injection of CO-saturated blood instead of by inhalation of the gas (60). The method may also prove useful in determining small amounts of CO in blood in chronic CO poisoning.

Extraction of gases from blood sample. In the chamber of the manometric apparatus 18 cc. of the acid ferricyanide solution and 6 or 7 drops of caprylic alcohol. The air is removed by evacuating, shaking three minutes, ejecting the extracted air and repeating the process once more. Six cubic centimeters of the solution are forced up into the cup of the apparatus. Then 5 cc. of blood are run under this layer of solution into the chamber of the apparatus from a pipette provided with a rubber tip, in the manner shown in figure 29 or 30. One cubic centimeter of the ferricyanide from the cup is then run down into the chamber to wash out the cock of the apparatus; the cock is sealed with a drop of mercury, and the 5 cc. of ferricyanide left in the cup are removed. The mercury in the chamber is now lowered to the 50-cc. mark and the evacuated chamber is shaken for seven minutes. Because of the large volume of solution a longer time than the usual two or three minutes is needed to obtain maximum extraction of the gases.

Reasons and precautions for the above steps are the same as for the oxygen determination described on p. 322.

Transfer of gases to Hempel pipette and absorption of CO_2 and oxygen. The leveling bulb is raised to such a height that the mercury surface in the bulb is a little above that in the chamber and the

extracted gases are collected in the top of the chamber under slight positive pressure. The stop-cock leading to the leveling bulb is closed, and the leveling bulb is left in the uppermost ring, above the chamber.

Before the Hempel pipette is used a little of the pyrogallate solution is run out to clear the stop-cock a (fig. 48) of any air that may be present. Then a little caprylic alcohol is put into cup *c*, and one drop is admitted into the capillary *r*. (The caprylic alcohol prevents foam formation in the pyrogallate solution.) The capillary limbs, *l* and *r*. are then filled with mercury from the cup *c* above. About 1 cc. of mercury is

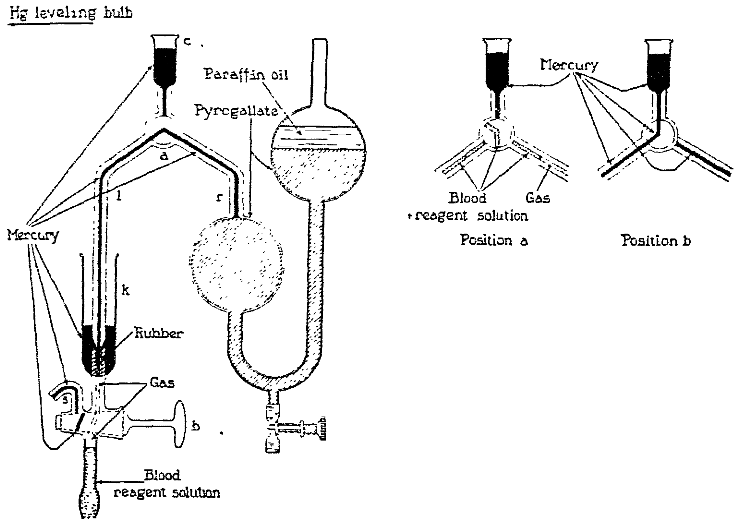


FIG. 48. Transfer of extracted blood gases to Hempel pipette in Sendroy-Liu determination of blood O_2 and CO. From Sendroy and Liu (32).

poured into the cup *k* of the Van Slyke-Neill apparatus, and all air is dislodged from the capillary leading down from the cup to the chamber.

The free end of the Hempel pipette, with mercury flowing through *l* from the cup *c* above, is thrust firmly down into the cup *k* so that the rubber tip fits snugly, as shown in figure 48, and stop-cock *a* is opened to the position indicated. (It is convenient to support the weight of the Hempel pipette by a wire or light chain.) Stop-cock *b* is then opened. At this point, if the internal pressure of the gas bubble has been correctly fixed, a small amount of the gas runs into the capillary limb of the Hempel pipette under its own pressure. The rest of the gas, followed

by the blood solution, is forced up into the Hempel pipette by admitting mercury slowly from the leveling bulb into the extraction chamber. As soon as the blood solution has passed slightly beyond the stop-cock *a*, the flow of mercury from the leveling bulb is stopped. Then cock *a* is turned in a clockwise direction to the closed position shown in figure 48, position *a*, and the Hempel pipette is withdrawn. In order to minimize the contamination of the absorbent by the blood solution, which precipitates in the pyrogallate and may even serve to trap gas, it is important to allow as little blood solution as possible to pass into the capillary limb *r*.

The free arm *l* is cleared of blood solution by the admission of mercury from cup *c*, and the capillary *r*, by continued turning of *a* in a clockwise direction, is likewise cleared of blood solution and gas. The pipette is set aside for the absorption of oxygen. Occasional gentle movement of the gas bubble to and fro, or in a horizontal rotatory manner accelerates the absorption, which is complete in three to four minutes. The octyl alcohol present in the pipette serves to prevent the gas from breaking up into small bubbles, and to increase the ease with which the bubble can be returned to the chamber.

Replacement of blood solution by air-free water in the extraction chamber. In the meanwhile the blood solution is removed from the chamber, which is then cleaned by alternate rinsing with dilute alkali and 1N sulphuric acid, followed by water. (Hyposulfite added to the alkali helps dissolve the methemoglobin particles.) Nine cubic centimeters of water are then measured from the cup into the chamber and rendered air-free by shaking in the evacuated chamber for two minutes. The extracted air is expelled, and 2 cc. of the water are run up into the cup *k*, 7 cc. remaining in the chamber.

Transfer of CO and N_2 from Hempel pipette to extraction chamber. One cubic centimeter of mercury is poured into the cup *k*, stop-cock *a* is turned counter clockwise, and the Hempel pipette is placed in position while mercury is flowing from cup *c* into cup *k* (figure 48, position *b*). Stop-cock *a* is then turned counter clockwise to the position shown in the main diagram of figure 48. The mercury leveling bulb is placed at its medium level, slightly lower than the chamber, and stop-cock *b* is opened. The stop-cock connecting leveling bulb and extraction chamber is carefully opened, and, by withdrawal of mercury from the bottom of the chamber, the gas bubble from the Hempel pipette followed by the drop of octyl alcohol is slowly drawn into the top of the chamber. The minimum possible amount of pyrogallate solution is allowed to flow

past the stop-cock *a*, which is then again turned back to the position indicated in figure 48, position *b*. The last portion of gas, with a slight amount of pyrogallate and some mercury from cup *c* is drawn into the chamber, which is then closed by the cock at its top.

The water level is lowered slightly below, then allowed to come to rest at, either the 2.0-cc. or the 0.5-cc. mark, according to whether much or little gas is present. A reading p_1 is taken.

Absorption of CO by Winkler's solution. Six cubic centimeters of air-free Winkler's reagent are placed in cup *k*. Of this, 5 cc. are slowly admitted into the chamber at slight negative pressure (leveling bulb as in figure 37). Because of the trace of pyrogallate which has followed the gas from the Hempel pipette into the chamber, the introduction of the first few drops of cuprous chloride solution causes a precipitate to form. This, however, upon further addition of the reagent, drops to the bottom of the liquid, leaving the top with a clear meniscus. The combination between CO and CuCl is a loose, reversible union. Consequently if after the absorption the CuCl solution were lowered in almost complete vacuum to the bottom of the chamber some of the absorbed CO would bubble out of the solution and return to the gas phase. The cuprous chloride is admitted slowly enough so that two minutes are taken. In this time absorption of carbon monoxide is complete. The surface of the solution is now lowered carefully to the original 0.5 or 2.0 cc. mark and the pressure p_2 in the manometer is read. The reading may be checked once by lowering the solution in the chamber a few millimeters and bringing it back to the mark. If several check readings are attempted, however, a gradual rise in the observed pressures will be noted, because the absorbed CO begins to return to the gas phase. The lowest reading of such a series is the correct one.

Some of the cuprous chloride may precipitate on the walls of the chamber. It is readily redissolved by a mixture of saturated NaCl and 1 N H_2SO_4 solutions.

Determination of c correction. Five cubic centimeters of water are extracted 1 minute in the apparatus to remove dissolved air, and the bubble of extracted air is ejected (for technique of ejection, see p. 279). The cock of the chamber is then sealed, and the meniscus of the water is lowered to the mark, either 0.5 or 2.0 cc., used in the CO determination. The pressure p_1' is read. Five cubic centimeters of air-free CuCl solution are then admitted and the reading p_2' is taken. The *c* correction is

$$p_1' - p_2' = c$$

Calculation. The pressure P_{CO} exerted by the CO gas at volume 2.0 or 0.5 cc. is calculated as

$$P_{CO} = p_1 - p_2 - c.$$

P_{CO} is multiplied by the factor in table 32 to obtain volume per cent of carbon monoxide in the blood.

Determination of CO and O_2 in 2-cc. blood samples

The technique is the same as in the above analysis, with the following exceptions.

Only 2 drops of octyl alcohol are required. The volume of acid ferricyanide solution extracted is 9 cc., of which 5 cc. are run up into the cup, and 1 cc. is returned to the chamber, so that the reacting mixture is 2 cc. of blood plus 5 cc. of ferricyanide solution.

After the gases have been extracted, and before they are transferred to the Hempel pipette, the CO_2 is absorbed with 1 cc. of air-free NaOH in the manner described for oxygen analyses. Pressure p_1 is read on the manometer, with the gases O_2, CO, and N_2 in the chamber at 2-cc. volume.

The gases are then transferred to the Hempel pipette, where O_2 is absorbed. In the meantime the blood mixture in the chamber is replaced by an equal volume (7 cc.) of air-free water, as described above.

The gases are returned to the chamber, and the pressure, p_2 is noted, with 2.0 cc. gas volume.

$$P_{O_2} = p_1 - p_2 - c_{O_2}$$

p_3 is then read, with the gases at 0.5-cc. volume.

The CO is then absorbed with cuprous chloride, as above described, and reading p_4, with the gas (N_2) at 0.5 cc. volume, is taken.

$$P_{CO} = p_3 - p_4 - c_{CO}.$$

c_{O_2} is obtained by a blank analysis without blood carried as far as the p_2 reading.

The value of c_{CO} is obtained as described for the preceding analysis.

The calculations are made by means of the factors in table 30.

Determination of CO_2, O_2, and CO in 1 cc. of blood

For this determination only 1 cc. of blood is used, since 2 cc. would be likely to yield too much total gas to be measured in the apparatus.

The steps are the same as in the preceding analysis, except in the details indicated below.

At the start 7.5 instead of 10 cc. of acid ferricyanide are placed in the chamber. The reaction mixture is 2.5 cc. of ferricyanide plus 1 cc. of blood.

One pressure reading, p_1, with 2.0 cc. gas volume is taken ***before*** the CO_2 is absorbed with NaOH.

The reading p_2 is taken at 2-cc. volume after absorption of CO_2 by NaOH.

The oxygen is then absorbed in the Hempel pipette, and the manometer reading p_3 is taken with the gases $CO + N_2$ at 2.0 cc. volume, and 3.5 cc. (instead of 7 cc.) of water in the chamber. The gas volume is then reduced to 0.5 cc. and reading p_4 is taken.

The CO is absorbed with cuprous chloride, and p_5 is taken at 0.5 cc. volume.

$$P_{CO_2} = p_1 - p_2 - c_{CO_2} \text{ (at } a = 2.0 \text{ cc.)}$$

$$P_{O_2} = p_2 - p_3 - c_{O_2} \text{ (at } a = 2.0 \text{ cc.)}$$

$$P_{CO} = p_4 - p_5 - c_{CO} \text{ (at } a = 0.5 \text{ cc.).}$$

c_{CO} and are c_{O_2} determined as for the preceding analysis.

To determine c_{CO_2} one extracts 2.5 cc. of acid ferricyanide and 1 cc. of water (in place of blood) in the chamber. The extracted gases are ejected and, with the meniscus in the chamber at the 2-cc. mark, the manometer is read before and after adding 1 cc. of 1 N NaOH. The fall in the reading (about 1 mm.) produced by adding the NaOH is the c_{CO_2} correction.

The calculations are performed with the factors in tables 28 and 30.

HEMOGLOBIN BY THE OXYGEN CAPACITY METHOD WITH SATURATION IN SEPARATE VESSEL (VAN SLYKE AND NEILL (59))

The blood is saturated with air as described for the same determination with the volumetric apparatus on page 265. The oxygen content is determined, as described above, and the oxygen bound by hemoglobin is estimated by substracting from the total O_2 0.5 volume per cent, or 0.22 millimol per liter, for the physically dissolved oxygen (see table 31).

The relative advantages of this method, compared with those in which the blood is saturated with air or carbon monoxide in the chamber of the

gas apparatus, are the same mentioned on page 265. The procedure for gasometric hemoglobin determination at present most in favor in the writers' laboratories when sufficient blood is available is to aerate 2 cc. of blood with a Stadie rotator (figure 34) in a 50-cc. centrifuge tube, and determine the O_2 content in samples of 0.5 cc. When less than 2 cc. of blood is available one of the methods described below is used.

HEMOGLOBIN BY OXYGEN CAPACITY METHOD WITH SATURATION IN THE CHAMBER OF THE GAS APPARATUS. SENDROY (31A)

When blood is diluted with water and hemolyzed the hemoglobin can not be completely oxygenated by saturation with air at ordinary pressure. However, if the blood is diluted with physiological saline solution the hemoglobin maintains its normal affinity for oxygen, and can be completely oxygenated by aeration. This fact has been utilized by Sendroy in devising a technique in which blood is both aerated and analyzed in the chamber of the Van Slyke-Neill apparatus. The correction for physically dissolved oxygen is quite large, but can be so accurately determined, or calculated from temperature and barometric pressure, that it introduces no significant error.

Reagents

Ferricyanide-saponin solution. (23 grams $K_3Fe(CN)_6$ and 8 grams saponin per 100 cc. solution).

1 N sodium hydroxide solution air-free. Described under "General reagents" on page 233–5.

Sodium hydrosulfite solution, air-free. Described under "General reagents" on page 233–5.

Isotonic sodium chloride solution (9 grams NaCl per liter solution).

Procedure for blood sample of 1 cc.

Aeration of blood. Two and five tenths cubic centimeters of 0.9 per cent NaCl are placed in the cup of the apparatus. Of this, 0.5 cc. is allowed to enter the chamber. One cubic centimeter of the blood sample is then delivered into the apparatus from a rubber-tipped pipette (fig. 29 or 30). The blood is completely washed in with the remaining 2.0 cc. of the salt solution, and the mercury level in the chamber is lowered to the 50-cc. mark with the stop-cock of the chamber ***open to the air.***

The blood-salt solution mixture is shaken with air at atmospheric pressure for three minutes, at the rate of 350 to 400 oscillations per

minute. The mixture is then run up to within a few centimeters of the stop-cock. One drop of octyl alcohol is added and is mixed with the fluid in the chamber by shaking the chamber a few times by hand. The air is then ejected in the way described on page 279.

Oxygen determination in aerated blood. In the manner shown in figure 52 (p. 344), 0.13 cc. of the ferricyanide reagent is added from a microburette to the blood mixture in the extraction chamber. The chamber is evacuated, the gases are liberated, and CO_2 and O_2 are absorbed in succession, as described on pages 284 and 322.

Calculation

$$\text{Volume per cent combined } O_2 \text{ in blood} = (p_1 - p_2 - c) \times \textit{factor}$$

p_1 is the manometer reading after adding NaOH to absorb CO_2.

p_2 is the manometer reading after adding hyposulfite to absorb O_2.

The *factor* is from table 30, p. 325 for "sample = 1 cc., S = 3.5 cc."

c is the $p_1 - p_2$ value obtained by readings before and after adding hyposulfite in the blank analysis. The c correction includes the following four components: 1, the O_2 dissolved by the 2.5 cc. of salt solution; 2, the O_2 in physical solution in the 1 cc. of blood; 3, a trace of O_2 added in solution in the 0.13 cc. of ferricyanide-saponin solution; 4, the fall (of about 1 mm.) of the mercury level in the chamber caused by adding 1 cc. volume of fluid with the hyposulfite.

The c correction can be determined by blank analysis in which 0.85 cc. of 0.9 per cent salt solution replaces the 1 cc. of blood. The blank analysis is done with 3.35 cc. of 0.9 per cent NaCl solution in place of 2.50 cc. of NaCl solution plus 1 cc. of blood. The assumption made by Van Slyke and Neill (59) is followed, that the 0.85 cc. of saline solution when aerated takes up in physical solution the same volume of O_2 as 1 cc. of blood. This assumption, if not absolutely exact, introduces no significant error, for oxygen capacities calculated with this correction have been found to agree closely with carbon monoxide capacities (31a, 56) by the method described below. The physically dissolved O_2 in the blood plus salt solution makes the greater part of the c correction, and varies significantly with temperature and barometric pressure, which influence the amount of oxygen dissolved when the blood-saline mixture is aerated. Hence if the empirically determined c correction is used, it must be redetermined with each set of analyses.

c correction calculated by nomogram. However, it is not necessary thus to redetermine the c correction. The physically dissolved O_2 in the aerated saline blood mixture (sum of components 1 and 2 enumerated above) is the

only part of the correction that varies measurably, and it can be accurately estimated from the solubility of oxygen in 0.9 per cent NaCl solution at the observed temperature and barometric pressure. The pressures exerted

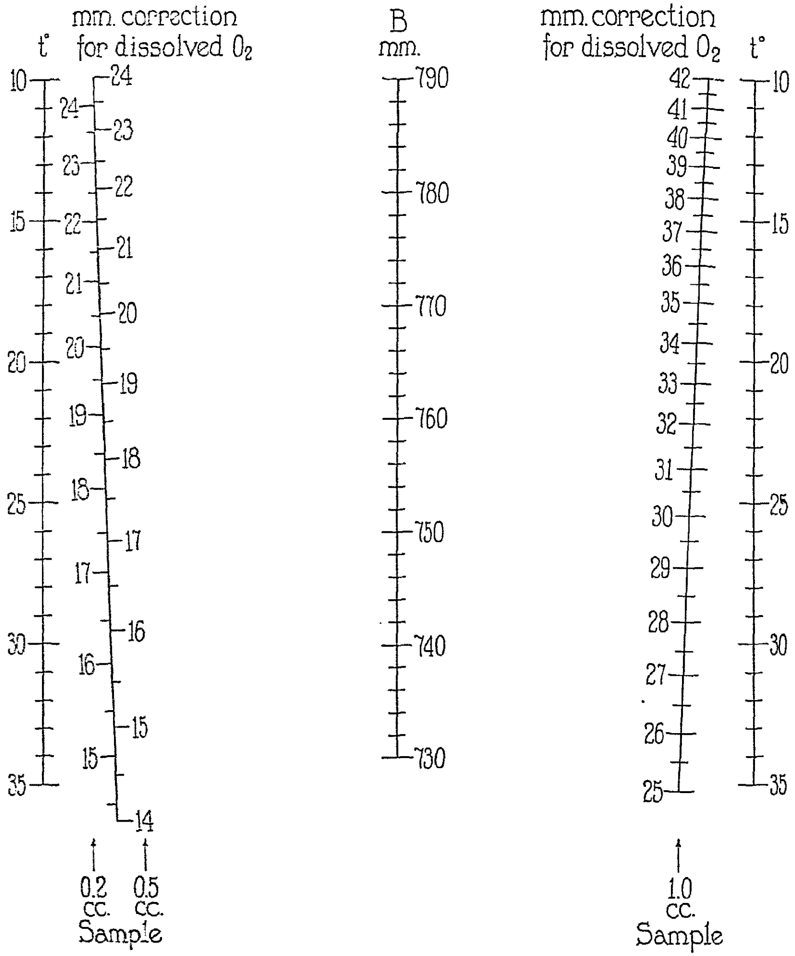


FIG. 49. Line chart to estimate correction for physically dissolved O_2 in Sendroy's method for hemoglobin determination by O_2 capacity. From Sendroy (31a).

at 2.0 and 0.5 cc. by the dissolved O_2 extracted from 3.5 cc. of air saturated 0.9 per cent salt solution are indicated by the line chart in figure 49. A straight line drawn across the temperature and barometer scales cuts the

central scale at a point indicating the pressure. To the pressure correction for dissolved oxygen thus obtained one adds a small constant correction for components 3 and 4 enumerated above, to obtain the total *c* correction. The correction for components 3 and 4 is obtained by a blank analysis in which 3.5 cc. of saline solution, in place of being shaken with air, are first extracted in the *evacuated* chamber to remove dissolved oxygen. The extracted air is ejected from the chamber (for technique see p. 279). The 0.13 cc. of ferricyanide is then added and the analysis is carried through as described above. The fall in pressure caused by addition of the hyposulfite is added to the correction for dissolved oxygen taken from figure 49 to obtain the total *c* correction. For a given apparatus the fall in pressure caused by addition of hyposulfite is constant. Consequently, the blank analysis described in this paragraph need be done only once. The *c* correction is thus obtained by adding *a* from the nomogram to *b*, the constant obtained by blank analysis. The value of *b* is about 2.5 mm. when measured with 0.5 cc. gas space in the chamber.

Procedure for blood samples of 0.2 or 0.5 cc.

One cubic centimeter of 0.9 per cent salt solution is placed in a small test tube of about 2 cc. capacity, or in the cup of the apparatus. The blood is drawn into a pipette calibrated to ***contain***, and is then delivered under the saline solution. The pipette is rinsed by drawing salt solution into it. The mixture of blood and salt solution is then run into the chamber of the apparatus. The cup (and the test tube, if one is used) is washed with saline solution, a few drops at a time, until the total volume of fluid in the chamber reaches down to the 2-cc. mark. Any saline solution left in the cup is removed. The blood mixture is then aerated in the chamber and analyzed for O_2, as described above for 1-cc. samples. To free the oxygen 0.07 cc. of ferricyanide-saponin solution is used, and for absorption of CO_2 and O_2, portions of 0.5 cc. of 1 N alkali and hyposulfite suffice. The *c* correction is determined as for 1-cc. samples, except for the difference in amounts of material used.

Calculation is as described above for 1-cc. samples, except that the factors and correction for 0.2 or 0.5 cc. are used.

HEMOGLOBIN BY THE CARBON MONOXIDE CAPACITY METHOD. VAN SLYKE AND HILLER (56)

This method is based on the capacity of hemoglobin to combine with the same maximum volume of CO as of O_2. The blood sample, which may

vary from 0.1 to 2.0 cc., is saturated with CO in the 50-cc. chamber of the manometric blood gas apparatus. For this purpose approximately 2 cc. of carbon monoxide gas are introduced with the blood, the mercury is lowered to the 50-cc. mark, and the chamber is shaken. The carbon monoxide tension of approximately 25 mm. is sufficient to change the hemoglobin quantitatively to HbCO, while O_2 and N_2 are at the same time completely extracted from the blood. The gases are ejected, and the HbCO is determined by measurement of the CO set free by acid ferricyanide solution.

The correction for physically dissolved O_2 is eliminated by this procedure. The substituted correction for physically dissolved CO is only 0.3 volume per cent, and variations in it are negligible so that it need be determined only once. With as little as 0.2 cc. of blood one can measure the CO binding capacity within ± 0.2 volume per cent, equivalent to 1 per cent of the amount of hemoglobin normally present.

Compared with the oxygen capacity, the carbon monoxide method has the advantages that it obviates the use of hyposulfite absorbent, and likewise the correction for dissolved oxygen. Also, if the blood contains any CO combined with its hemoglobin as drawn, such CO causes no error in the CO capacity method, whereas it would cause low results by the O_2 capacity methods. The relative disadvantage of the carbon monoxide method is that it requires the use of an extra reagent, the CO gas.

Reagents

Special acid ferricyanide solution. To 92 volumes of stock solution containing 32 grams of $K_3Fe(CN)_6$ per 100 cc. are added 8 volumes of concentrated lactic acid, of specific gravity 1.2. This concentrated acidified solution undergoes very slow decomposition, but can be used for 2 months.

Air-free 1 N sodium hydroxide solution. Already described under "General reagents."

Approximately 5 N sodium hydroxide. Described under "General reagents" in this chapter.

Carbon monoxide gas. This is prepared by warming a mixture of anhydrous formic and sulfuric acids. According to the reaction, $HCOOH = CO + H_2O$, each cubic centimeter of formic acid yields about 500 cc. of CO. In figure 51 is shown a simple arrangement for preparing and storing 3 liters of the gas, enough for 1500 analyses. The two 5-liter aspirator bottles are connected by a rubber tube, the bore of which should be as wide as 15 mm. in order to assure a flow of water sufficiently free to prevent the development of back pressure during generation of the CO. Before the latter process is begun the bottle A is filled completely with water. Sulfuric acid is dropped

slowly into the formic acid in the tube, which is gently warmed with a micro burner. When about 300 cc. of mixed air and CO from the test-tube have collected in *A* the gases are ejected by opening the outlet cock of *A* and raising *B*. Then the rest of the sulfuric acid is run slowly into the formic acid, and the reaction is continued until the CO from all the formic acid has been collected in *A*. The rubber tube connecting the test-tube with *A* is then closed with a screw clamp close to the glass inlet tube of *A*, and the test-tube is disconnected from *A*. *Because of the toxicity of CO the above operation should be carried out in a hood or where there is a free draft of air.*

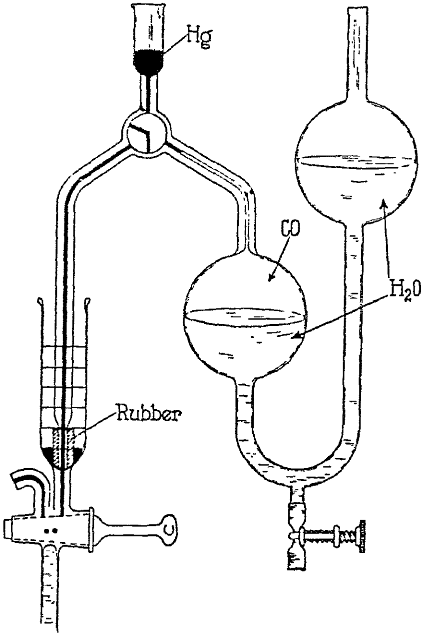


FIG. 50. Introduction of CO into manometric chamber in hemoglobin determination by carbon monoxide capacity method. From Van Slyke and Hiller (56). (For construction of the Hempel pipette, see figure 11 of chapter III.)

Procedure for analysis of 2 cc. blood samples

Measurement of blood sample into apparatus. One drop of caprylic alcohol is drawn into the capillary beneath the cup of the manometric apparatus. (This capillary should be of only 1-mm. bore.) Into the cup are measured 4.75 cc. of water. With a pipette, provided with a rubber tip (see figure 29 or 30), 2 cc. of blood are run directly into the chamber, followed by the 4.75 cc. of water.

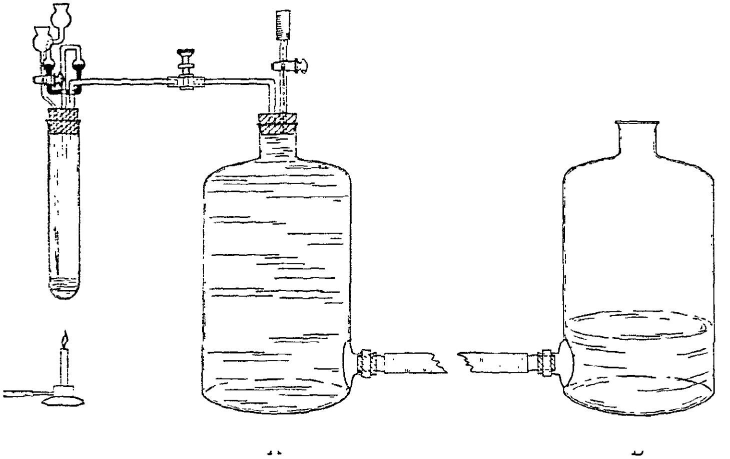


FIG. 51. Apparatus for preparation and storage of carbon monoxide. From Van Slyke and Hiller (56).

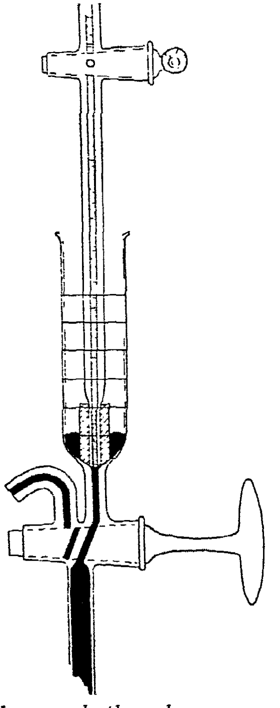


FIG. 52. Transfer of solution sample through mercury seal to chamber of blood gas apparatus. When the pipette or burette tip is withdrawn a droplet of solution floats up on the mercury and is lost, but the amount is only 0.005 to 0.01 cc. From Van Slyke (48).

Measurement of CO into chamber of apparatus. Approximately 2 cc. of CO, measured at atmospheric pressure, are required. This amount provides 0.50 cc. to combine with the maximum amount of hemoglobin ordinarily found in human blood, and leaves an excess of 1.5 cc., which is sufficient, when extended to 43 cc. of volume during the equilibration, to leave a CO pressure of about 25 mm. and insure quantitative change of Hb to HbCO. The exact amount of CO may vary from 2 cc. to 3 cc. if it is accurately duplicated in the blank analyses in which the correction for physically dissolved gases is determined. Any convenient device may be used for measuring the CO. The following has been found satisfactory.

One or 2 cc. of mercury are placed in the cup above the chamber. The outlet capillary of the Hempel pipette is filled with mercury. The tip of the Hempel pipette is then fitted into the bottom of the cup, as shown in figure 50. The two cocks shown in the figure are now turned so that the CO gas can flow from the Hempel pipette into the chamber of the Van Slyke-Neill apparatus. The flow is regulated, not by either of the cocks shown, but by the cock leading to the mercury leveling bulb of the manometric apparatus. With the leveling bulb in the mid-position shown in figure 37 this cock is opened slowly, and mercury is withdrawn from the chamber until CO gas has entered it as far as the 2-cc. mark. The gas is thus measured under a pressure indicated by the difference in height between the water columns in the two bulbs of the Hempel pipette. This difference may vary from about 10 cm. of water when the pipette is full of gas to 0 cm. when it is nearly empty. Such variation, however, amounts to only 0.01 of an atmosphere, which is negligible.

Equilibration of blood solution with CO. Both blood solution and CO gas having been measured into the chamber, the cock of the latter is closed and is sealed with a drop of mercury, and the chamber is evacuated. With the mercury meniscus at the 50-cc. mark, the chamber is shaken until equilibrium is reached. The time required varies somewhat with the speed of the motor. The necessary time is found by equilibrating for different periods in the blank analyses described below, and finding how long one must shake the chamber in order to reduce the blank to a nimimum. Ordinarily this time is one minute. Equilibration being completed, the mixture of free gases, composed of all the O_2 and N_2, and part of the CO_2 from the blood, together with the excess CO, is ejected from the chamber. (See section on "Ejection of gas without loss of solution" on page 279.)

Determination of CO bound as HbCO. After ejection of the free gases the chamber is evacuated until the blood solution is in the lower fourth, in order to keep the calibrated upper portion clean when the acid ferricyanide to be added precipitates methemoglobin. About 1 cc. of mercury and 2 or 3 cc. of water are placed in the cup. Through the mercury seal 0.25 cc. of the acid ferricyanide solution is added from a micro burette graduated into 0.01-cc. divisions, and provided with a rubber-tipped delivery capillary, as shown in figure 52. Before the tip of the burette is inserted into the mercury it is moved through the supernatant layer of water, which dislodges any adherent ferricyanide crystals or air bubbles.

After addition of the ferricyanide only enough mercury is admitted into the capillary beneath the cup to fill that capillary and the bore of the cock. It is preferable to have no mercury globules run down into the chamber at this time, because they are likely to adhere to the film of ferricyanide on the walls of the upper 2 cc. of space, which it is desirable to keep free and clean for subsequent gas measurement.

To extract the CO set free from HbCO by action of the ferricyanide, the mercury in the chamber is lowered to the 50-cc. mark, and the chamber is shaken for about five seconds slowly, to avoid too rapid foaming, then vigorously for three minutes.

Mercury is now admitted from the leveling bulb into the chamber until the gas space above the solution is reduced to 5 or 6 cc., and the CO_2 in the gas phase is absorbed with 1 cc. of air-free 1 N NaOH in the manner described for CO_2 determinations.

The volume of the remaining gas, CO, with a slight trace of air introduced with the ferricyanide, is brought to 2 cc. and the pressure observed on the manometer is recorded as p_1. The gas is ejected without loss of solution (see page 279). The stop-cock of the chamber is again sealed with a drop of mercury and the fluid meniscus is lowered to the 2-cc. mark. The manometer reading now observed, with the chamber gas-free, is recorded as p_2.

Calculation

The hemoglobin content of the blood in terms of CO or O_2 binding capacity is calculated by the equation

$$\text{CO or } O_2 \text{ capacity} = (p_1 - p_2 - c)$$

f is a factor from table 30 and c is a correction, determined by blank analysis,

for the slight amounts of CO physically dissolved by the equilibrated blood solution, and of air admitted in solution in the 0.25 cc. of ferricyanide.

The value of c is determined by a blank analysis in which the procedure described above is repeated in every detail, except that 2 cc. of water are substituted for the 2 cc. of blood. The correction c is calculated as

$$c = p_1 - p_2$$

With a temperature of 20° to 25° and barometric pressure of 740 to 780 mm., the value of c is about 4.0 mm., of which 1.4 mm. are due to air admitted in the ferricyanide and 2.6 mm. to CO physically dissolved by the equilibrated blood solution.

The cleaning of the chamber, after each analysis, is performed by rinsing with three successive portions of water, as described on page 236. To the first water portion a few drops of alkaline hyposulfite solution are added to dissolve particles of methemoglobin adherent to the walls of the chamber.

Procedure for analysis of 1-cc. blood samples

The procedure is the same as when 2 cc. of blood are used, except that 2.5 cc. of water are taken instead of 4.75 cc., 0.13 cc. of the acid ferricyanide solution is used to liberate the CO, and 0.5 cc. of air-free 1 N sodium hydroxide is used to absorb the CO_2. The amount of CO added is the same, 2 cc. measured at atmospheric pressure. The c correction must be determined with these amounts of reagents. With blood of less than ordinary hemoglobin content, more exact results will be obtained by using the 0.5 cc. mark for the gas reading.

Procedure for analysis of 0.1, 0.2, or 0.5-cc. blood samples

The blood is drawn into a capillary pipette calibrated to contain either 0.1 or, preferably, 0.2 cc.

If the blood sample is drawn outside the laboratory, the pipette is delivered into a small test-tube of about 2-cc. capacity, containing 0.5 cc. of water for a 0.1-cc. blood sample, 1.0 cc. of water for 0.2-cc. or 0.5 cc. The pipette is rinsed twice by drawing up into it the supernatant water. The blood and water in the tube are then mixed with a fine rod or wire.

Before the sample thus diluted is transferred to the Van Slyke-Neill apparatus a drop of caprylic alcohol is drawn into the capillary below the cup of the apparatus. The blood solution from the test-tube is transferred to the cup and thence, with the drop of caprylic alcohol,

down into the chamber. The test-tube and cup are washed with repeated water portions of 4 or 5 drops each, until the volume of solution inside the chamber extends to the 2 cc. mark.

In the same manner described above for analysis of 2-cc. blood samples, 2 cc. of CO gas are added and equilibrated with the blood solution.

Of the ferricyanide solution only 0.07 cc. is added. It is run in while the blood solution is still in the top of the chamber, and is followed by several drops of mercury, which break up the methemoglobin precipitate into fine particles.

The chamber is evacuated and shaken three minutes. The fluid is permitted to rise as near to the cock at the top of the chamber as it will with the leveling bulb at the mid-position (fig. 37). Three or 4 drops of 5 N sodium hydroxide are run into the chamber, followed by a few drops of mercury. The alkali absorbs the CO_2 gas, and, mixed by the falling mercury with the dilute blood solution, dissolves the suspended methemoglobin particles. The aqueous meniscus is lowered below the 0.5 cc. mark and then brought slowly up to it.

After the p_1 reading is taken, the gas is ejected, the cock is sealed with mercury, the meniscus is again lowered to the 0.5 cc. mark, and p_2 is read on the manometer.

The correction, c, is determined by repeating the analysis without addition of blood.

The calculation factors for 0.2 and 0.5-cc. samples are given in table 30. When a 0.1-cc. sample is used the factor for 0.2 cc. is multiplied by 2.

STANDARDIZATION OF STANDARD SOLUTIONS FOR PALMER HEMOGLOBIN METHOD. (VAN SLYKE AND HILLER (56A))

The Palmer method for colorimetric determination of hemoglobin after it is transformed into HbCO is described in chapter 20. The stock standard solution, which is made by approximately 5-fold dilution of blood of normal hemoglobin content, must be saturated with pure carbon monoxide gas instead of illuminating gas if it is to be standardized by the method here described.

Reagents

The reagents are the same as those described for the above carbon monoxide capacity method with the exception of the acid ferricyanide solution.

This is made by mixing 92 volumes of a solution containing 32 grams of $K_3Fe(CN)_6$ per 100 cc. with 52 volumes of concentrated lactic acid of specific gravity 1.2. Although slow decomposition takes place, such a solution not exposed to direct sunlight can be used for two months.

Procedure

Two drops of caprylic alcohol are drawn into the capillary beneath the cup of the manometric apparatus. In the cup is placed about 1 cc. of mercury. With a stop-cock pipette provided with a rubber tip (fig. 52), 10 cc. of the Palmer hemoglobin standard are run into the chamber under the mercury. Through the same mercury seal 2 cc. of CO are added as described in the carbon monoxide capacity method. The procedure continues as described above for that method, except that 0.36 cc. of the present acid ferricyanide reagent is used to liberate the CO bound as HbCO, and 1.5 cc. of air-free 1 N NaOH are used to absorb the CO_2. The absorption of CO_2 takes somewhat longer, hence it is important to note the fall of mercury in the manometer. When the mercury ceases falling, agitate the chamber slightly with the hand. If no further change occurs in the manometer meniscus the absorption is completed.

The *c* correction is established by carrying out the procedure in the same manner, except that 10 cc. of an aqueous solution containing 4 cc. of strong ammonia per liter are substituted for the hemoglobin standard. This correction is ordinarily about 7 or 8 mm. Each analyst should establish his own *c* correction repeatedly.

Calculation is the same as that for the carbon monoxide capacity method for 2-cc. blood samples.

METHEMOGLOBIN. VAN SLYKE AND HILLER (57)

The principle of the method was first employed by Nicloux and Fontes (24). Two determinations are required. In one (*A*) *the normal or active form of hemoglobin*, capable of binding O_2 and CO, is determined by measuring the CO-binding capacity of the hemoglobin-methemoglobin mixture. In the other (*B*) sodium hyposulfite is added, changing methemoglobin into active reduced hemoglobin, and the *total hemoglobin* is determined by the CO-binding capacity. *The difference, B–A, indicates the methemoglobin.* The technique for the CO capacity determinations is that described above. All the operations, reduction with hyposulfite, saturation with CO, and de-

termination of CO bound by hemoglobin, are carried out in the chamber of the manometric apparatus.

In determining the carbon monoxide capacity in blood reduced by hyposulfite, the only modification necessary to the carbon monoxide capacity procedure for hemoglobin determination is the use of higher CO tensions to saturate the hemoglobin. The presence of hyposulfite and ammonia appears to lower somewhat the affinity of reduced hemoglobin for CO, so that 100 mm. tension of the latter, instead of only 25, are required to insure complete conversion of the hemoglobin to carboxyhemoglobin. To insure a sufficient excess of CO, an initial tension of 150 mm. is used.

Reagents

Nicloux's ammoniacal sodium hyposulfite solution. In a 100 cc. beaker place 2 grams of pulverized sodium hyposulfite, $Na_2S_2O_4$. In another beaker mix 50 cc. of water and 1 cc. of concentrated ammonia solution. Pour the diluted ammonia solution over the hyposulfite and cover the mixture at once with a layer of paraffin oil about 1 cm. deep, to prevent oxidation by air. Dissolve the hyposulfite by stirring for a few seconds with a rod. Commercial hyposulfite usually contains some insoluble impurity; consequently to attempt to stir until complete solution is attained is useless, and would cause oxidation of part of the hyposulfite.

The *carbon monoxide gas, 1 N air-free sodium hydroxide,* and *5 N sodium hydroxide* are prepared and handled as in the carbon monoxide capacity method described above.

The *acid ferricyanide solution for use with the blood portion to which ammonia and hyposulfite have been added,* is prepared as follows, with more acid than for the CO capacity method. To 92 volumes of a stock solution containing 32 grams of $K_3Fe(CN)_6$ per 100 cc. are added 20 volumes of concentrated lactic acid, of specific gravity 1.2. The ferricyanide in this acidified solution undergoes slow decomposition, but if kept out of direct sunlight can be used for about 2 months.

Procedure

Determination of active hemoglobin. The carbon monoxide capacity method previously described is used without change.

Determination of total hemoglobin. For 2 cc. of blood. Two drops of caprylic alcohol are drawn into the capillary beneath the cup of the manometric apparatus. Into the cup are measured 4.3 cc. of water. With a pipette provided with a rubber tip (see figure 29 or 30) 2 cc. of

blood are run directly into the chamber, followed by a few drops of the water in the cup to wash the blood through the capillary. From a microburette 0.4 cc. of the ammoniacal sodium hyposulfite solution is run into the chamber, by the technique shown in figure 30, and is followed by the remaining water in the cup. One or 2 cc. of mercury are placed in the cup.

Carbon monoxide sufficient to give 150 mm. of pressure is measured into the chamber from a modified Hempel pipette by the following procedure described on page 111 for "Admission of sample estimated by pressure."

The equilibration of the blood solution with CO is carried out as described in the carbon monoxide capacity method except that a little more time seems necessary, about one and one-half minutes instead of one minute.

The determination of CO bound as HbCO is also carried out in all details, including the c correction, as described in the carbon monoxide capacity method, except that 0.3 cc. instead of 0.25 cc. of acid ferricyanide solution is added. The value of c in the present procedure is somewhat greater than in the carbon monoxide capacity method because of the greater amount of CO physically dissolved by the blood solution at the higher CO pressure used for saturation. The value of c found by Van Slyke and Hiller (57) with a temperature of 20–25° and the gas at 2 cc. volume was about 14 mm. Each analyst should, however, determine it for himself.

The cleaning of the chamber after each analysis is more important in this case than in the simple carbon monoxide capacity determination, because in the present procedure any particle of methemoglobin precipitate left adhering to the walls of the chamber will be reduced in the next determination to active hemoglobin by hyposulfite, and added to the total hemoglobin found. A little of the hyposulfite solution added to the first portion of water used to clean the apparatus assists in dissolving such particles quickly.

For 1-cc. blood samples. The procedure is the same as that used for 2-cc. samples except that half as great a volume of each reagent is used for 1 cc. of blood as for 2 cc. The pressure of CO used is the same, 150 mm.

Micro determination of total hemoglobin with 0.1-cc. or 0.2-cc. blood samples. The procedure for measuring the blood and transferring it to the chamber of the apparatus is the same as that described in the carbon monoxide capacity method except that 0.05 cc. of

the ammoniacal sodium hyposulfite solution is run into the chamber of the apparatus before the final washing of the cup, so that this volume of fluid is part of the total 2 cc. measured into the chamber. Carbon monoxide to 150 mm. pressure is admitted into the evacuated chamber in the same manner described above. Of the acid ferricyanide solution only 0.05 cc. is added. It is run in while the blood solution is still in the top of the chamber and is followed by several drops of mercury which break up the methemoglobin precipitate into fine particles. The procedure is continued as described in the carbon monoxide capacity method.

The c correction for the micro method is somewhat over 20 mm. It must be redetermined with each set of micro analyses.

Calculation of methemoglobin

The calculations for total hemoglobin are the same as those described for the carbon monoxide capacity method.

$$\text{CO capacity} = (p_1 - p_2 - c)f$$

Calculation factors are found in table 30.

(Methemoglobin) = (total hemoglobin) − (active hemoglobin)

GASES IN BLOOD CONTAINING ETHER. AUSTIN (2)

When blood contains ether in amounts (100 to 180 mg. per 100 cc.) required to produce deep anesthesia, ordinary procedures for the determination of blood gases are invalidated, because considerable amounts of ether vapor become mixed with the extracted gases. Furthermore, Austin (2) found that alkali added to absorb CO_2 also absorbs part of the ether. Consequently, CO_2 determinations by the ordinary technique applied to the blood of etherized subjects may give results 10 to 15 volumes per cent in excess of the true values.

However, Austin has shown that correct CO_2 values can be obtained if, after absorption of CO_2, the alkaline blood solution is again extracted *in vacuo* so that the ether absorbed by the added alkali solution is partly returned to the gas phase. The following technique is employed.

The blood is acidified with lactic acid and extracted in the usual manner in the chamber of the manometric apparatus. The manometer

reading p_1 is taken and the extraction is repeated. If p_1 increases, extraction is again repeated.

The CO_2 is then absorbed with alkali and p_2 is read as usual. The mercury in the chamber is then dropped again to the 50-cc. mark and the chamber is shaken for three minutes. The gas volume is then reduced to 2 cc. and p_3 is read on the manometer. The extraction and reading are repeated in order to make sure that a constant p_3 has been reached. The CO_2 content is then calculated as:

$$[CO_2] = [p_1 - p_3 + k\ (p_3 - p_2)] \times f$$

$[CO_2]$ = CO_2 content of the blood; f is the factor from table 28 for calculating CO_2 content. The value of k is determined empirically by mixing with a standard sodium carbonate solution proportions of ether, such as 1 or 2 mg. per cubic centimeter, of the order of magnitude encountered in the blood analyzed. The carbonate solution is then analyzed in the special manner above indicated, and the value of k is calculated by the above equation rearranged into the form:

$$k = \frac{[CO_2] - f(p_1 - p_3)}{f(p_3 - p_2)}$$

In this case $[CO_2]$ is known from the Na_2CO_3 content of the standard carbonate solution.

In Austin's analyses the volume of acidified solution extracted was 2.5 cc. (instead of the 3.5 cc. now extracted when 1 cc. of blood is analyzed), and the CO_2 was absorbed with 1 cc. of N sodium hydroxide. Under these conditions, and at temperatures in the neighborhood of 25°, the value of k was 0.13. Analysts who use Austin's method of correction, should, however, determine k for themselves for the conditions under which their analyses are made.

Thus far no one has devised a technique for determining the O_2 content of blood containing ether, although it could presumably be done by a procedure similar to that of Austin for CO_2.

Manometric Analyses Other Than Blood Gas Determinations

MICRO-KJELDAHL DETERMINATION OF NITROGEN. VAN SLYKE (47)

Principle

The ammonia in the Kjeldahl digest is estimated gasometrically by treatment with hypobromite in the manometric apparatus of Van Slyke and Neill.

The pressure of 1 mg. of nitrogen measured at 2-cc. volume in the apparatus is about 300 mm. so that accurate readings are easy: duplicate results usually check to within 1 per cent.

The nitrogen gas evolved by the reaction with hypobromite,

$$2\ NH_3 + 3\ NaBrO = 3\ H_2O + 3\ NaBr + N_2$$

falls somewhat short of that calculated by the above equation, but the percentage of the theoretical yield is so constant under defined conditions of hypobromite and alkali concentration that, with the introduction of an empirical correction factor, the error due to variability in this reaction may, without special precautions, be kept within 0.5 per cent of the ammonia determined.

The only difficulty in devising an accurate microgasometric technique was encountered in finding a satisfactory general catalyzer for the digestion which would not prevent subsequent reaction of the ammonia with hypobromite, nor cause formation of gases other than the nitrogen evolved from the ammonia. Various catalysts and oxidizers previously employed for Kjeldahl determinations were tested. Copper salts were found by Stehle (39) to react with hypobromite and evolve oxygen gas. Mercury forms a combination with ammonia which resists the action of hypobromite (47). Perchloric acid if used in excess destroyed part of the ammonia during the digestion. Hydrogen peroxide gave inconstant results. However, when potassium persulfate in great excess as used by Wong (67) was tried, satisfactory results were obtained with regularity. The digestion even of protein material is completed in a few minutes, and with less resistant material such as urine or blood filtrates oxidation is completed so quickly that the dark color of carbon does not even appear. Furthermore, persulfate does not destroy any ammonia. Unnecessary prolongation of the digestion consequently does not injure the results, provided, of course, the overheating is not carried to the extreme of volatilizing ammonium sulfate.

About the only point that requires especial consideration when persulfate is used is the water content of the digest at the time the persulfate is added. If the material to be analyzed is dissolved in several cubic centimeters of water per cubic centimeter of acid used, and sulfuric acid and persulfate are both added before the water is boiled off, all of the persulfate will decompose during the subsequent boiling down and before the solution has become concentrated enough for digestion to occur. Results will accordingly be low because of lack of oxidizing agent during the actual digestion. This was pointed out by Wong (67). On the other hand, if the sulfuric acid solution

is boiled down till SO_3 fumes come off, and then dry persulfate is added, the latter will not be completely decomposed during the digestion and will evolve appreciable amounts of oxygen when treated with hypobromite. The mixture of ordinary concentrated sulfuric acid and syrupy phosphoric acid, 3:1, which was finally adopted, appears to have enough moisture in it to act with the persulfate on a dry substance; and 1 cc. of water is not too much. The procedure adopted for samples dissolved in more than 1 cc. of water is accordingly to boil them down with 1 cc. of the acid mixture till fumes begin to come off, and to add 1 gram of persulfate, with 1 cc. of water, to the cooled residue, before finishing the digestion.

The use of phosphoric acid in the digest is not necessary, but it accelerates the digestion. 0.1 cc. of blood serum digested with 1 cc. of sulfuric acid and 1 gram of persulfate requires about fifteen minutes to clear. If a mixture of 1 part phosphoric to 3 parts of sulfuric acid is used, instead of sulfuric alone, five minutes suffice. A larger proportion of phosphoric acid is likely to attack the glass tubes.

The hypobromite solution used was an object of considerable experiment. Various concentrations of bromine in various concentrations of alkali have been used in the past. All of them form oxygen while standing. Stehle's solution (39), containing per 100 cc. 2.5 grams of Br, 2.5 grams of KBr, and 5.3 grams of NaOH, was found to form oxygen several times as fast as solutions of bromine in more concentrated NaOH without bromide. The solution finally adopted, 1 cc. of bromine to 50 cc. of 40 per cent sodium hydroxide, generates oxygen so slowly that the amount formed in an hour in 1 cc. of the solution (the amount used for an analysis) exerts at 2 cc. volume only about 0.3 mm. pressure. The presence of 40 grams of NaOH per 100 cc. reduces the solubility of air in the solution to so nearly zero that it is not necessary to extract the air before the solution is used, the slight trace of air present being included in the blank for the reagents. The amount of bromine present in 1 cc. of this solution is sufficient to decompose in one minute the maximum amount of ammonia for which the method is designed, and it is not enough to cause trouble by the rate of its attack on the mercury in the gas apparatus.

Reagents

Sulfuric-phosphoric acid mixture. Three volumes of concentrated sulfuric acid are mixed with 1 volume of syrupy phosphoric acid (specific gravity 1.7).

Potassium persulfate containing less than 0.01 mg. of ammonia nitrogen per gram.[8]

Forty per cent sodium hydroxide. Forty grams of NaOH per 100 cc. of solution, ammonia-free.

Sodium hypobromite solution. To 50 cc. of the 40 per cent NaOH in a 250-cc. flask add, under a hood, 1 cc. of bromine, and stir until the bromine is completely dissolved. Gaseous oxygen forms very slowly in this solution. In order to prevent even slight accumulation, however, the hypobromite is aerated by whirling it about the walls of the flask for a few seconds each time before a portion is used for analysis. *The hypobromite solution is made fresh on the same half day in which it is used.*

Procedure

Digestion. In a 200 by 25 mm. test-tube of Pyrex glass or silica place the sample to be analyzed, containing preferably 0.3 to 1.5 mg. of nitrogen, 1 cc. of the sulfuric-phosphoric acid mixture, and a glass bead to prevent bumping. If the volume of water added with the sample is 1 cc. or less, 1 gram of potassium persulfate is added at once. If a larger volume of water is present, however, it is first boiled down till white fumes appear, the mixture is cooled and 1 gram of persulfate is then added, together with 1 cc. of water. Heating is continued until the mixture has become entirely clear, or after white fumes appear in the case of substances which oxidize so readily that the digest is clear throughout the period of heating. A micro burner is used, and during the final heating somewhat less heat is used than in the ordinary micro-Kjeldahl-Gunning digestion. The tip of the burner should just touch the bottom of the test-tube. An unnecessary intensity or prolongation of the heating may cause bumping, or corrosion of the test-tube by

[8] *Purification of potassium persulfate.* Ordinary commercial $K_2S_2O_8$ contains much ammonia. It may be purified as follows. The persulfate is dissolved in 10-fold its weight of water previously warmed to 60° to 70°. The solution, made strongly alkaline to litmus by addition of 40 per cent NaOH, is transferred to a vacuum distilling apparatus, and distilled under a pressure of less than 40 mm. When about one-third of the solution has been distilled off, it is replaced by an equal volume of water at 60° to 70°, more alkali is added if necessary to restore alkalinity to the solution, and the distillation is continued until the distillate tested by Nessler's reagent is ammonia-free. The addition of warm water and alkali may have to be repeated again before all the ammonia is removed. When ammonia has been completely removed, the persulfate solution is poured into a beaker and let stand overnight, preferably in an ice box, to crystallize. The crystals are washed with cold water on a Buchner funnel, and dried over sulfuric acid in a vacuum desiccator. Persulfate prepared this way can be obtained from Merck and Company.

the phosphoric acid in the digestion mixture. Enough heat may be used towards the end of the digestion to fill the tube with white fumes, but not enough to drive them out of the tube.

Occasionally a substance unusually difficult to digest (e.g., a lipoid) may fail to clear in fifteen minutes. In such a case the digest is cooled somewhat, and 0.2 to 0.3 gm. more of potassium persulfate with 2 or 3 drops of water is added. Digestion is then continued.

Neutralization of digest and transfer to gas apparatus. The digest is cooled, 3 cc. of water are poured upon it, and a drop of 1 per cent alizarin sulfonate is added. From a pipette 40 per cent sodium hydroxide is then added, a drop at a time with occasional *cooling of the tube* in a cold water stream. The heat of the reaction causes solution of the solidified melt in the bottom of the tube. Addition of alkali is continued until the mixture becomes just alkaline to the alizarin. Ten per cent sulfuric acid is then quickly added till the color changes back to acid, in order to prevent loss of ammonia.

The solution, cooled to room temperature, is poured into the cup of the Van Slyke-Neill blood gas apparatus and the volume of the solution is noted. The solution is then drawn down into the chamber. Into a graduated pipette is drawn enough water to make the total volume of the solution up to 10 to 11 cc., and this water is used in three portions to rinse into the gas apparatus the drops of solution left in the test-tube.

Gasometric determination of ammonia nitrogen. The cock of the gas chamber is sealed with a drop of mercury, the chamber is evacuated and is shaken two minutes to extract the air from the solution. The extracted air bubble is ejected by the technique described on page 279 for ejection of gases from chamber without loss of solution. The extraction is repeated, yielding usually a very small second air-bubble, which also is ejected without permitting more than a drop of the solution to follow it up into the cup of the apparatus.

The hypobromite solution is whirled for a few seconds about the walls of its flask to permit escape of any oxygen gas that has formed in it, and 1.5 cc. are transferred to the cup of the gas apparatus. One cubic centimeter is run down into the chamber. The remaining 0.5 cc. is washed out of the cup, and the cock is sealed with mercury. The chamber is then evacuated and shaken two or three minutes.

The gas volume is reduced to 2 cc. and the reading, p_1, on the manometer is noted, together with the temperature.

The gas is then ejected from the chamber, the meniscus of the solution is lowered again to the 2-cc. mark, and p_2 is read on the manometer.

When sufficient material for duplicate analyses is available, one may take an amount of material containing 1 to 4 mg. of nitrogen, with 2 cc. of sulfuric-phosphoric acid mixture and 2 grams of persulfate. The digestion is carried out in a tube marked at 25 cc. capacity. After neutralization the digest is diluted up to the mark, and 10 cc. aliquot portions are taken for gasometric nitrogen determinations.

TABLE 33

FOR MICRO-KJELDAHL ANALYSIS. FACTORS BY WHICH MILLIMETERS OF NITROGEN PRESSURE MEASURED AT 2-CC. VOLUME ARE MULTIPLIED TO OBTAIN MILLIGRAMS OF NITROGEN* (FROM VAN SLYKE (47))

TEMPERATURE	FACTOR	TEMPERATURE	FACTOR
°C.		°C.	
15	0.003250	25	0.003136
16	41	25	24
17	27	27	16
18	15	28	04
19	01	29	3092
20	3192	30	80
21	80	31	71
22	68	32	60
23	56	33	50
24	45	34	40

* These factors include the empirical correction factor of 1.04, for the reaction with hypobromite.

Calculation

$$\text{Milligrams of } N = (p_1 - p_2 - c) \times f$$

where f is the factor indicating the mg. of N corresponding to 1 mm. of pressure at the temperature noted. The factor is found in table 33.

$$p_1 - p_2 - c = \text{pressure of nitrogen gas from the substance analyzed}$$

c is the value of $p_1 - p_2$ obtained in a blank analysis of the reagents, which are digested with 1 cc. of water and otherwise treated as in the analysis described. With good reagents the blank, c, does not exceed 6 mm.

Under the conditions given, the hypobromite yields 96 per cent of the theoretical amount of nitrogen gas. Consequently the factors of table 33 give the weight of N_2 measured in the apparatus multiplied by 1.04.

In order to obtain the factors in table 33, the factors in table 30 marked "Sample = 1 cc., S = 3.5 cc., a = 2.0 cc., i = 1.00" were multiplied by

$$\frac{28\ 08}{1000} \times 1.04 = 0.0292$$

Applications to blood and urine analysis

For determination of the total nitrogen of serum it is convenient to dilute 10-fold and digest 1 cc. of the solution, equivalent to 0.1 cc. of serum.

For albumin nitrogen in the filtrate obtained by Howe's method, in which 0.5 cc. of plasma is added to 15 cc. of 22 per cent Na_2SO_4, 5 cc. of the filtrate are boiled down with 1 cc. of the sulfuric-phosphoric acid mixture till fumes appear. The mixture is cooled. One cubic centimeter of water and 1 gram of potassium persulfate are added, and the digestion is continued as usual. Because of the sodium sulfate present it is well to add 5 cc. of water, instead of 3 cc., before neutralizing to avoid crystallization of the salt in the tube. The final volume of fluid transferred to the gas apparatus is therefore 13 to 14 instead of 10 to 11 cc., but the results are not affected.

Non-protein nitrogen of blood is done on 10 cc. of trichloroacetic acid or Folin-Wu filtrate, which is boiled down in the tube with 1 cc. of sulfuric-phosphoric acid mixture before adding the persulfate plus a few drops of water.

Urines if of ordinary concentration are diluted 5-fold, if unusually concentrated, 10-fold, and 1 cc. samples of the solution are digested.

AMMONIA

The technique described above for determining the ammonia formed by Kjeldahl digestion can be used in general for micro determinations of ammonia in solutions that are free from interfering substances.

When a definite volume of ammonia-containing solution is measured into the gas chamber one may, instead of extracting the air as in the above described micro Kjeldahl analysis, leave the air in and measure it with the the ammonia, the pressure of the two together giving the reading p_1 on the manometer. A blank analysis of the reagents at the same temperature gives reading p_0. Then

$$p_1 - p_0$$

This procedure necessitates but one manometer reading with each ammonia solution; and in a series of analyses the gas chamber need not be washed out between analyses, since the trace of hypobromite left after ejecting one solution will do no harm in analysis of the next. It is assumed that p_0 observed in the blank analysis represents the pressure arising from both ammonia and air in the reagents. The procedure can be used therefore only if the dissolved air in the solution submitted to blank analysis is the same as in the ammonia solution. Both solutions must be saturated with air at within 0.2° of the same temperature, and both must contain like amounts of salts, alkali, or other solute affecting the solubility of air. In case the temperature rises or falls between the readings of p_0 and p_1, p_0 must be corrected by adding or subtracting, respectively, the change in vapor pressure of water caused by such a temperature change (see table 18, p. 163).

Whether it is more desirable to remove dissolved air in each analysis or include a correction for it in a p_0 reading must be decided by the analyst in accordance with the conditions. The analysis with preliminary removal of air is about five minutes longer, but is free from the slight sources of error, due to possible failure of blank and ammonia solutions to have quite the same solubility for air, and to possible slight errors in making the corrections for temperature changes between the p_0 and p_1 readings. If a considerable series of analyses is to be done, with amounts of ammonia nitrogen in the neighborhood of 1 mg., it will probably be worth while to use the more rapid method, with p_0 readings from blank analyses. If, however, only a few analyses are intended, or if the determinations are on small fractions of a milligram of ammonia, the preliminary extraction of dissolved air, and calculation of P_{N_2} as in the micro Kjeldahl method will be desirable.

When air is extracted before addition of hypobromite, as in the Kjeldahl analyses, the solution must be *acid* or ammonia will be lost.

UREA BY MEASUREMENT OF THE CARBON DIOXIDE FORMED BY THE ACTION OF UREASE. VAN SLYKE (49)

Principle

Urea is changed by the action of urease[9] into ammonium carbonate: $CO(NH_2)_2 + 2\ H_2O = (NH_4)_2CO_3$. The ammonia has been commonly determined as a measure of the urea. However, as shown by Partos (27) and by Mirkin (22), one can also obtain an exact result by determining the

[9] For experiments and literature concerning the mode of action of urease and the optimum conditions for its action see papers by Van Slyke and Cullen and Van Slyke and Zacharias cited in the general chapter on urea determination.

CO_2 of the ammonium carbonate. The manometric blood gas apparatus is particularly adapted to this determination, because of the wide range over which it yields accurate results. In practice the gasometric CO_2 urea determination has proved to have several advantages over the ammonia estimation. The gasometric method dispenses with the apparatus required for aeration or distillation of the ammonia, and with the necessity for exact standard solutions for titration, or for colorimetric comparison in Nesslerization. The result is a diminution in the sources and likelihood of error, and, at least in urine analyses, a gain in rapidity.

Preformed carbonic acid and bicarbonate exist in both blood and urine. This CO_2 is removed by acidifying and agitating the blood or urine before the urease is added.

The removal of the blood proteins is not a necessary preliminary to the analysis. The determination can be performed directly on either whole blood or plasma, as well as on the Folin-Wu blood filtrate. The only drawback to the direct analysis of whole blood is that the determination requires 15 minutes, compared with 5 for the Folin-Wu filtrate.

In accuracy there is no difference between determination of the CO_2 and that of the NH_3 formed by the action of urease, if samples of size best suited for the measurement of each can be taken. In urine analyses this is the case. In blood analyses the results obtainable with 1-cc. samples by the gasometric method are about equal in accuracy (1 per cent of the amount measure) to those obtainable with 5 cc. by the ammonia titration method, and considerably more accurate than those obtainable by titration with the 2-cc. samples commonly used. Gasometrically one can obtain with a little practice satisfactory accuracy, by the microtechnique described below, with 0.2 cc. of blood taken from an ear puncture.

Urea in urine

Reagents

Urease solution. A 10 per cent water solution of Squibb's urease, which is prepared from jack beans by Van Slyke and Cullen's acetone precipitation method (52). It has always been found highly active and free of CO_2. Any other CO_2-free urease preparation may presumably be used, if the activity (see p. 377), or the time required by it to decompose completely the maximum amount of urea that may be encountered in the analysis undertaken, is determined. The urease solution in 50 per cent glycerol prescribed for blood analysis may also be used for urine. See "Urease preparations," chapter 11.

1 N lactic acid (approximate). Described in the section of this chapter on "General reagents."

2 M H_3PO_4 (approximate). Dilute 13.2 cc. of syrupy phosphoric acid, of specific gravity 1.725, to 100 cc.

Saturated carbonate-free NaOH solution, approximately 18 N, described in the section of this chapter on "General reagents."

2 M carbonate-free NaOH. Dilute 11 cc. of the saturated sodium hydroxide to 100 cc. At once draw the solution up into a tube (fig. 53) protected from atmospheric CO_2 by soda-lime at the top.

Brom-thymol blue, 0.4 per cent solution. 100 mg. of the powdered dye are ground in a mortar with 3 cc. of 0.05 *N* NaOH, and then diluted to 25 cc. with water.

Caprylic-ethyl alcohol. 1 volume of caprylic alcohol is mixed with 4 volumes of 95 per cent ethyl alcohol. This mixture is used to prevent foaming. For use with urine it is preferable to pure caprylic alcohol, because it is less likely to obscure the clear meniscus.

Test of 2 M H_3PO_4 and 2 N NaOH

A portion of 2 M H_3PO_4 is titrated with the 2 N NaOH, using phenolphthalein as indicator and titrating to a full red. Each cubic centimeter of phosphoric acid should neutralize 2 cc. of sodium hydroxide. A variation of 10 per cent may be permitted, i.e., 1 cc. of H_3PO_4 may neutralize from 1.80 to 2.20 cc. of NaOH. It is convenient for the titration to dilute 3 cc. of the 2 M H_3PO_4 with 20 cc. of water, and to add the alkali from a 10 cc. burette.

The 2 N NaOH should be titrated as above when it is made up and once a week thereafter. If the solution stands for some weeks or months in the soda-lime tube it is likely to increase in concentration by more than the permitted 10 per cent, as the result either of losing water by slow distillation up into the soda lime, or of solution of more alkali from the glass. An intact coat of paraffin on the tube should prevent reaction with the glass. It is advisable even in a paraffin-coated tube, however, to test alkali that has stood for some time.

Procedure for urea determination in urine

Removal of preformed CO_2. **Place in a 25-cc.[10] measuring flask 2 cc. of urine of specific gravity below 1.030, or 1 cc. of more concentrated**

[10] In the original description of the method (49) a 20-cc. flask was used. We have changed to a 25-cc. one for the reason that 20-cc. flasks are not stock articles, and consequently are likely to be missing from laboratory equipment unless especially ordered for this analysis. Table 34 has been recalculated from the original (49) to provide for the change in final dilution.

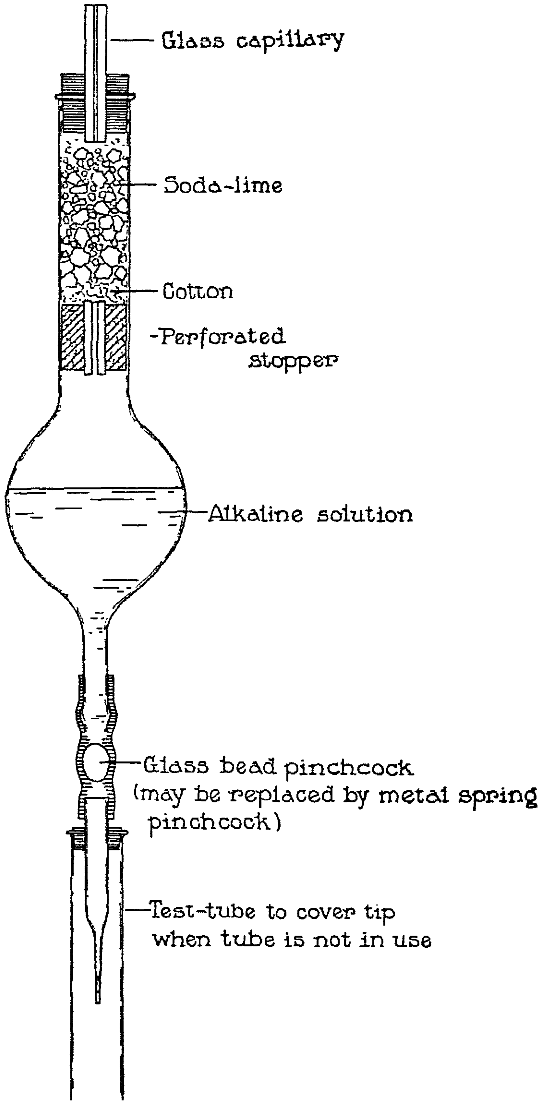


FIG. 53. Soda lime tube of about 100-cc. capacity for holding CO_2-free alkali or alkaline phosphate. The tip is drawn out quite fine, so that it delivers 35 to 40 drops per cubic centimeter, the exact number being determined by trial with the solution used, and marked on the tube. The form of cock shown, made by a glass bead in a rubber tube, is convenient for delivery of drops by count. By pinching the tube about the bead delivery of the fluid is made at the desired rate. An ordinary metal pinch-cock may also be used. From Van Slyke (49).

urine. Add 0.25 cc. of 2 M H_3PO_4, which may be measured conveniently and with sufficient accuracy by drops delivered from the fine glass tip of a dropping pipette. Then add 3 drops of brom-thymol blue solution. Whirl the urine about the walls of the flask for one minute to permit CO_2 to escape. Insert a tube into the flask and draw air through it to sweep out the CO_2 gas. Then whirl the urine again for a minute to permit escape of the last traces of CO_2. (This second treatment is necessary only for urines with such high bicarbonate content that they are alkaline

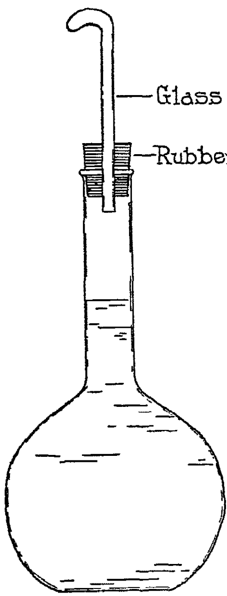


FIG. 54. Volumetric flask for preparation of urine for urea determination with urease. From Van Slyke (49).

to litmus, but it is probably safer to carry it through as a routine procedure on all.)

Digestion of urine with urease. Dilute to about 23 cc. Add 0.35 cc. of 2 N NaOH, measured by drops from the tube in which it is stored. (The first drops from the tube are discarded, as the solution at the capillary tip is likely to have taken up CO_2 from the air.) Then add 1 cc. of urease solution from a pipette which dips below the surface of the diluted urine. The heavy urease solution sinks to the bottom of the flask and does not decompose the urea at the top until after the solutions

are mixed. The solution is now diluted up to 24.9 cc., and the flask is closed with a 1-hole rubber stopper, the hole of which is filled by a vaselined glass rod (fig. 54). The contents of the flask are mixed, and

TABLE 34

UREASE METHOD FOR UREA IN URINE

Factors by which millimeters of pressure from urea CO_2 are multiplied (from Van Slyke (49))

Volume of solution extracted = 3.5 cc. Gas volume at which pressure is measured = 2.000 cc.

TEMPERATURE	FACTORS GIVING GRAMS OF UREA N PER LITER URINE		FACTORS GIVING GRAMS OF UREA PER LITER URINE	
	Sample = 1 cc. urine	Sample = 2 cc. urine	Sample = 1 cc. urine	Sample = 2 cc. urine
°C.				
15	0.04304	0.02154	0.0922	0.0461
16	4278	41	18	59
17	55	27	13	56
18	32	16	07	53
19	10	05	02	51
20	4189	2095	897	49
21	66	85	92	46
22	42	74	88	44
23	21	63	84	42
24	01	52	79	39
25	4081	42	75	37
26	62	31	71	35
27	43	21	66	33
28	24	12	62	31
29	04	02	58	29
30	3985	1993	54	27
31	68	84	50	25
32	50	75	46	23
33	33	66	42	21
34	15	58	39	19

"Sample" signifies volume of urine measured into 25 cc. flash. Only 2/25 of the sample is taken for the gasometric determination.

allowed to stand 20 or more minutes for the enzyme to act. The mixed solution should take on a distinctly green color, and if much urea is present will quickly become blue from formation of ammonium car-

bonate. If when the urine solution is mixed with the added alkali and urease the color does not change from yellow to at least green (pH about 6.5), add another drop of 2 N alkali through the hole in the stopper. This may be necessary with an occasional urine of high acidity.

Addition of excess alkali to digested urine to prevent CO_2 loss. At the end of the twenty minutes or longer period the glass rod is withdrawn from the stopper and, after the first 3 drops of alkali have been discarded, the capillary tip of the tube (figure 53) delivering 18 N NaOH is inserted through the hole. 0.2 cc. of the concentrated alkali is added, measured by (about 5) drops. It is advisable to vaseline the delivery tip, so that the alkali will fall cleanly from the end and not creep back up to the sides. The 0.2 cc. of concentrated alkali increases the volume of the solution by only 0.1 cc. The stopper is again closed with the glass rod, and the flask is inverted 2 or 3 times to mix the alkali with the solution and to absorb any CO_2 that may have escaped into the gas space beneath the stopper. The 0.2 cc. of concentrated NaOH makes the solution so alkaline that there is no danger of loss of CO_2 when the flask is subsequently opened for withdrawal of samples. To prevent absorption of atmospheric CO_2 the flask is kept closed, except when samples are pipetted out for analysis.

Extraction and measurement of CO_2 formed from urine urea. Place a drop of the caprylic-ethyl alcohol mixture in the capillary and 1 cc. of water in the cup of the manometric apparatus. Run 2 cc. of the digested urine solution under the water. Admit the urine solution into the chamber of the apparatus followed by the 1 cc. of water. Then add 0.5 cc. of 1 N lactic acid. Evacuate the chamber and shake 1.5 minutes. Reduce the gas volume to 2 cc. as described for CO_2 determinations in blood. Read pressure p_1 on manometer, and eject the solution from the apparatus.

Run a control with reagents alone, without any urine. The manometer reading for the control is p_0.

Calculation

The pressure due to CO_2 from urea is

$$P_{urea} = p_1 - p_0$$

P_{urea} is multiplied by the proper factor from table 34.

One control serves for an entire series of urea determinations. If the temperature changes while the series is being analyzed, correct p_0 by adding or

subtracting 1.3 mm. for each degree rise or fall of temperature centigrade. This serves to correct for the change in the vapor tension of the water in the apparatus and in the pressure exerted by the air extracted from the solution, if the temperature does not vary more than 3°. If it does, repeat the analysis of the control solution.

Remarks on the urine analysis

In running a series of analyses it is not necessary to wash the gas apparatus between determinations. Since the CO_2 is not reabsorbed with alkali in the apparatus, the ejection of the gas and the acidified solution after each analysis removes the CO_2 with sufficient completeness. One may consequently proceed with the analyses at the rate of one every four minutes, and have sufficient leisure to calculate each result during the extraction in the next analysis.

The 1 cc. of water and the 0.5 cc. of lactic acid solution added to the urine solution in the apparatus should be measured to within 0.05 cc. The dissolved air in the 3.5 cc. of solution is extracted and measured with the CO_2, the correction for the air content being included in the blank. 0.1 cc. of water saturated with air at room temperature yields sufficient air to exert at 2 cc. volume about 1 mm. pressure in the apparatus, and it is desirable to keep the error within this limit. Hence the 3.5 cc. volume of water solution should be measured to within 0.05 cc. The 1 cc. of water first placed in the cup should be measured from a 1-cc. pipette, and the 0.5 cc. of lactic from a 0.5-cc. pipette, rather than by the marks on the cup itself.

Urea determination in Folin-Wu blood filtrate

The determination may be made, as in the urine analysis described above, by measuring the total gas (air + CO_2) after the action of urease, and subtracting the dissolved air obtained in a blank analysis. This procedure (*A*) is the one of choice for speed and convenience when a number of blood urea determinations are required; each determination is simplified to one extraction and one reading of the manometer, and as the CO_2 is not reabsorbed with alkali, the apparatus need not be washed between determinations. For the blank analysis, 0.9 per cent NaCl solution is used, because it dissolves the same amount of air as the Folin-Wu filtrate, viz., 94.5 per cent as much as does water.

When only one or two analyses are to be made, it is simpler to omit the blank and perform in the gas apparatus the extraction of preformed CO_2 as well as the subsequent analysis. Accordingly both procedures will be described.

Reagents for blood filtrate analysis

0.9 per cent NaCl solution, acidified with 1 or 2 drops of 1 N hydrochloric or sulfuric acid per 100 cc.

0.5 M CO_2-free Na_2HPO_4. 17.9 grams of $Na_2HPO_4 \cdot 12\ H_2O$, or 7.1 grams of anhydrous Na_2HPO_4, made up to 100 cc. To avoid absorption of atmospheric CO_2 the solution should be made with minimum exposure to air and at once drawn up into a soda-lime tube (fig. 53).

Another way of preparation, from the H_3PO_4 and concentrated NaOH described for urine analysis, is also convenient. In a 100-cc. measuring flask place about 50 cc. of water and 3.33 cc. of syrupy H_3PO_4 of specific gravity 1.72 (this is approximately 15 M H_3PO_4). Add 1 drop of phenolphthalein and run in carbonate-free 18 N NaOH till the solution turns pink. At once dilute to 100 cc., mix, and draw up into a soda-lime tube.

10 per cent urease in glycerol-phosphate solution. Jack bean urease dissolved in water slowly produces carbon dioxide, sufficient in the course of an afternoon to increase by several millimeters the pressure reading in a blank analysis. When the enzyme is dissolved in 50 per cent glycerol CO_2 formation does not occur. Phosphate also is added to the solution to neutralize the acidity of the Folin-Wu filtrate and provide a proper pH for the action of the urease.

Place 1 gram of Squibb's urease in a 10 cc. measuring cylinder and stir into it 2 cc. of water. When the mixture has become homogeneous add 3 cc. of the 0.5 M Na_2HPO_4 solution, followed by 5 cc. of glycerol. Mix, and at once draw up into a 10 cc. burette protected at the top by a soda-lime tube. The delivery tube of the burette below the cock should be 7 or 8 cm. long, and should be provided with a rubber tip to permit delivery through a mercury seal directly into the chamber of the gas apparatus as shown in figure 52.

5 N NaOH solution and 1 N lactic acid (approximate). Described in the section of this chapter on "General Reagents." The 5 *N* NaOH is drawn into a soda-lime tube (fig. 53).

0.4 per cent brom-thymol blue, described above for urine analyses.

Caprylic-ethyl alcohol mixture, 1 to 4, described for urine analyses.

Procedure A, for determinations in a series of blood filtrates

Preliminary aeration of filtrate and control solution. **Prepare the filtrates by treatment of the blood with tungstic acid, according to Folin and Wu, as described in chapter II. The blood is diluted 10-fold in the process. Collect each filtrate in a flask of volume equal to at**

least 10-fold that of the filtrate and add a drop of 0.4 per cent solution of brom-thymol blue for each 10 cc. The filtrate must show a clear yellow acid color. If there is any tinge of green a drop of 0.1 N acid is added. To remove preformed CO_2 derived from the blood bicarbon-

TABLE 35

UREASE METHOD FOR UREA IN FOLIN-WU BLOOD FILTRATE

Factors by which millimeters of pressure from urea CO_2 are multiplied (from Van Slyke (49)).

Volume of solution extracted (S) = 5.7 cc. Sample = 0.5 cc. blood. $\alpha' = 0.945\ \alpha'$ for water.

TEMPERATURE	FACTORS GIVING MILLIGRAMS OF UREA N PER 100 CC. BLOOD		FACTORS GIVING MILLIGRAMS OF UREA PER 100 CC. BLOOD	
	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.
°C.				
15	0.720	0.1834	1.540	0.393
16	15	21	30	90
17	10	09	20	88
18	06	0.1798	10	85
19	01	87	00	83
20	0.697	76	1.491	80
21	93	65	82	78
22	88	54	73	76
23	84	43	64	73
24	80	33	55	71
25	76	23	47	69
26	72	13	39	67
27	68	03	31	65
28	65	0.1694	23	63
29	61	85	15	61
30	58	76	07	58
31	54	68	00	57
32	51	59	0.1393	56
33	48	51	87	54
34	45	43	80	52

To calculate grams per liter (= mg. per cc.) each factor is divided by 100.

ate, shake each flask, ***unstoppered***, vigorously with a horizontal, whirling motion from thirty seconds to one minute. Insert a tube into the flask and pass fresh air through it for a few seconds. Repeat this process twice more. Each extraction removes about 90 per cent of the CO_2

present, so that the last leaves only a negligible thousandth of that originally present. Several flasks may be held in the hand and shaken together when a series of analyses is being performed.

For a blank analysis treat a similar volume of acidified 0.9 per cent NaCl solution in the same manner, with the precaution to ascertain that the solution is *within 0.2°C of the temperature of the filtrates.* A variation of 1° would change the dissolved air content by 0.003 volume per cent, and affect the obtained blood urea nitrogen content by 0.5 mg. per 100 cc.

Transfer of sample to gas apparatus and digestion with urease. With a 5-cc. stop-cock pipette, provided with a rubber tip and calibrated to deliver between two marks (see fig. 30), transfer 5 cc. of filtrate to the chamber of the blood gas apparatus. Before the transfer a small drop of caprylic-ethyl alcohol is drawn into the capillary below the cup, into which are then poured 3 or 4 cc. of water and 0.5 to 1 cc. of mercury. The tip of the delivering pipette is sealed by immersion in the mercury, as shown in figure 52, so that the solution is delivered from the pipette directly into the chamber of the gas apparatus. It is convenient, though not obligatory, to use a pipette provided with a stopcock.

0.5 cc. of the urease solution, measured from the burette, is then run into the chamber under the mercury seal in the same manner. Before the burette tip is lowered into the mercury it is washed in the water in the cup to remove the drop of urease-phosphate at the tip, which may have absorbed some CO_2 from the air. The urease and filtrate are mixed by lowering and raising the mercury in the chamber a few centimeters, and are permitted to react for one or more minutes. One minute is sufficient with the Squibb's urease recommended above, of which 100 mg. at pH 6.8 to 7.2 and a temperature of 20° will decompose 9.5 mg. of urea per minute. The mixture should turn to a greenish color. If it remains yellow, because of unusual acidity of the filtrate, another 0.5 cc. of urease-phosphate solution may be added.

Determination of CO_2 formed from urea. After the urease has acted 0.20 cc. of 1 N lactic acid is run under the mercury seal into the chamber from a rubber-tipped burette in the same manner as the sample and urease. The chamber is evacuated and shaken one and one-half minutes. The gas volume is reduced to 0.5 cc. for ordinary blood, but only to 2.0 cc. for blood with high urea content. The technique described on page 277 for adjusting the gas volume in CO_2 determinations is to be followed. In case one attempts first to reduce the

volume to 0.5 cc., but finds the gas pressure too high to read in the manometer, the mercury in the chamber must be lowered again to the 50-cc. mark, the solution shaken for another half minute, and the gas volume brought to 2.0 cc. With the gas volume at either 0.5 or 2.0 cc. the pressure p_1 is read on the manometer.

Five cubic centimeters of 0.9 per cent NaCl solution are analyzed in the same manner. The reading is p_0.

The pressure of CO_2 from urea is $P_{urea} = p_1 - p_0$ as in the urine analysis described above.

In case the temperature recorded by the thermometer in the gas apparatus changes during the interval between the analysis of the blank and that of the filtrate, 1.5 mm. is added to p_0 as in the urine analysis for each 1° rise when the pressure is measured with the gas at 2-cc. volume. When the pressure is measured with the gas at 0.5 cc. volume the correction is 2.0 mm. per 1° temperature change. This correction is added to p_0 if the filtrate is analyzed with the gas apparatus at a higher temperature than the blank, and subtracted if the reverse occurs.

Procedure B, for determinations in single samples of blood filtrate

Removal of preformed CO_2. Five cubic centimeters of filtrate preceded by a drop of caprylic-ethyl alcohol are delivered into the gas apparatus under a mercury seal as described above. To remove CO_2 the chamber is evacuated and shaken thirty seconds. Mercury is admitted from below until the chamber is about one-quarter full. Then, ***without stopping the inflow of mercury, the upper cock is opened, admitting air into the chamber.*** The admission of mercury into the chamber is continued until all the gases have been completely driven out through the cock above. The air is admitted in order to dilute the CO_2 in the chamber and prevent its reabsorption by he blood solution. If the upper cock were kept closed until the extracted CO_2 has been compressed at the top of the chamber, some of the extracted CO_2 gas would go back into solution. The above procedure removes 90 per cent of the preformed CO_2 from the blood. It is repeated twice, making three extractions in all, which leave only a negligible 0.001 of the preformed CO_2.

Digestion with urease. 0.5 cc. of the urease-phosphate solution is measured into the cup of the apparatus, through a mercury seal, as in procedure ***A***. The urease is mixed with the filtrate in the chamber by once lowering the mercury to the bottom of the chamber. The mixture is allowed to stand 1 minute or longer for completion of the action of the

urease. It should turn from clear acid yellow to a green or blue color, indicating that the phosphate has neutralized the acidity of the filtrate. If the solution does not turn green, more urease-phosphate solution is added.

Determination of CO_2 formed from urea. 0.20 cc. of 1 N lactic acid is added through the mercury seal; the gases are extracted and their pressure, p_1, read at either 0.5 or 2.0 cc. volume, as described above for procedure *A*.

To absorb the CO_2, 5 to 6 drops of 5 N NaOH are admitted into the chamber. The cock leading to the leveling bulb is then opened to permit the solution to rise for a moment into the upper stem of the chamber. The gas volume is brought again to the original 0.5 to 2.0 cc., and the manometer reading p_2 is taken. The pressure P_{urea} due to CO_2 formed from urea is

$$P_{urea} = p_1 - p_2 - c$$

where c is the value of $p_1 - p_2$ obtained in a blank analysis performed on 5 cc. of acidified water in the place of blood filtrate. The c value, due to traces of CO_2 in the enzyme and phosphate solution, should be only a few millimeters with the gas volume at 0.5 cc.

Washing the apparatus between analyses. Before each analysis the gas apparatus is washed twice with 5 to 10 cc. of distilled water, as described in the section on general gasometric technique above.

Direct urea determination in whole blood or plasma

As in procedure *B* described above for blood filtrate, the entire operation, including removal of preformed CO_2 and digestion with urease, is carried out in the gas apparatus. Two procedures will be described, for the analyses of 1.0 and 0.2-cc. samples respectively.

Reagents for direct analysis of whole blood or plasma

18 N and 5 NaOH, 1 N and 0.1 N lactic acid described under "General reagents."

0.02 N lactic acid (for micro blood analysis). The 0.1 N acid is diluted 5-fold.

0.26 M Na_3PO_4 solution. In a 100 cc. flask place 1.75 cc. of syrupy phosphoric acid of specific gravity 1.72 to 1.73. This phosphoric acid is of approximately 15 M H_3PO_4 concentration. Fill the flask half full with water, add 2 drops of 1 per cent phenolphthalein solution and titrate with the carbonate-free saturated (approximately 18 N) NaOH solution from a 10

cc. burette until the solution in the flask turns red. At this point 2 molecules of NaOH have been added per molecule of H_3PO_4 and Na_2HPO_4 has been formed. Note the volume of NaOH solution used and add exactly half as much more, to change the phosphate to Na_3PO_4. The total volume of 18 N NaOH required should be about 4.3 cc. Immediately after the addition of the alkali has been completed, and before the alkaline phosphate solution has had opportunity to absorb CO_2 from the air, dilute the solution to the 100-cc. mark, stopper the flask, mix the solution, and draw it up into a soda-lime tube (fig. 53), from which it may be measured by drops for analyses.

Ten per cent urease solution in 50 per cent glycerol. One gram of Squibb's preparation of urease is dissolved in 5 cc. of water, and 5 cc. of glycerol are mixed with the solution. The enzyme solution should be prepared the same day it is used. It need not be protected from air, as it is too acid to absorb CO_2. It is not expedient to mix it with the Na_3PO_4 prior to use, because the alkalinity of the phosphate destroys the enzyme rather rapidly. Sometimes the glycerol as purchased contains dissolved CO_2, which causes a high blank. The CO_2 can be removed by distributing the glycerol in a thin layer about the walls of an evacuated flask.

Determination in 1 cc. of whole blood or plasma

Removal of preformed CO_2 from blood. Place a drop of caprylic ethyl alcohol mixture in the cup of the blood gas apparatus and draw it down into the capillary beneath the cup. Then place 1 cc. of 0.1 N lactic acid in the cup, and add water up to the 4.5 cc. mark. From a stop-cock pipette run 1 cc. of blood under the water solution into the chamber and then draw the acidified water also into the chamber. If a bubble of air enters the chamber no harm is done; it will be ejected later with the preformed CO_2. The preformed CO_2 is now removed by three successive extractions of 30 seconds each, in the manner described above for Procedure *B* with the Folin-Wu filtrate.

Digestion of blood with urease. After the removal of preformed CO_2, place in the cup of the apparatus 1 cc. of 10 per cent urease solution, and run into the urease solution 0.25 cc. of the 0.26 M Na_3PO_4 solution, measured by drops from the soda-lime tube (the first 2 or 3 drops are to be discarded as they have absorbed atmospheric CO_2). The urease-phosphate mixture is at once drawn down into the chamber of the apparatus and the cock is sealed with a drop of mercury. In order to mix urease and blood, and to bring the enzyme into contact with the portions of blood solution wetting the walls of the chamber, the mercury

in the chamber is lowered to the bottom and then permitted to rise again. The mixture is now permitted to stand a sufficient length of time (one minute with Squibb's urease) for the enzyme to complete its action.

Extraction and measurement of CO_2 formed from blood urea. After the enzyme has finished its action 0.5 cc. of 1 N lactic acid is placed in the cup and 0.25 cc. is drawn down into the apparatus, making the total volume of solution up to 7 cc. The cock is sealed with mercury, the chamber is evacuated and is shaken two minutes, as in determinations of blood CO_2. The volume of the extracted gas is reduced, ordinarily to 0.5 cc. However, if the blood urea content is over 75 mg. per 100 cc. (urea nitrogen over 35 mg.), the CO_2 pressure at 0.5 cc. volume will exceed 400 mm. With such bloods the volume of the extracted gas is reduced only to 2 cc. The technique described on page 277, for adjusting the volume of extracted CO_2 for pressure measurement is followed.

With the extracted gases at 0.5 or 2 cc. volume, the manometer reading p_1 is noted. Without releasing the vacuum, 3 or 4 drops of 5 N NaOH solution are admitted, a drop at a time, from the cup to absorb the CO_2. The vacuum is then released and the solution is permitted to rise for a moment into the upper stem of the chamber, to wash out the alkali. The mercury in the chamber is finally lowered again until the surface of the blood solution has fallen to the 0.5 or 2 cc. mark used for the first reading, and the second manometer reading p_2 is taken.

A *blank analysis* is carried through, in which the blood is replaced by water solution. The difference between p_1 and p_2 in the blank analysis is the correction, c, for CO_2 in reagents. The value of c is usually 5 or 6 mm. with a gas volume of 0.5 cc., and 1 or 2 mm. with a gas volume of 2 cc. The pressure due to CO_2 from urea in the analysis is

$$P_{urea} = p_1 - p_2 - c$$

The CO_2 pressure c observed in the control analysis is due in part to a trace of CO_2 in the alkaline phosphate, in part to the trace of CO_2 present in the water used to dissolve the urease.

A total solution volume as great as 7 cc. for 1 cc. of blood is used because the 5 N sodium hydroxide can be run into it for absorption of CO_2 at the end of the analysis. If only a 3-fold dilution of whole blood were used, the 5 N alkali would cause a gummy precipitate of hemoglobin on the walls of the

chamber. One would have to use 1 N alkali, which would have to be freed of air.

TABLE 36

UREASE METHOD FOR UREA IN WHOLE BLOOD OR PLASMA

Factors by which millimeters of pressure from urea CO_2 are multiplied (from Van Slyke (49)).

TEMPERATURE	FACTORS TO GIVE MILLIGRAMS OF UREA N PER 100 CC. BLOOD			FACTORS TO GIVE MILLIGRAMS OF UREA PER 100 CC. BLOOD		
	Sample = 1 cc. blood		Sample = 0.2 cc. blood	Sample = 1 cc. blood		Sample = 0.2 cc. blood
	a = 0.5 cc. S = 7.0 cc.	a = 2.0 cc. S = 7.0 cc.	a = 0.5 cc. S = 2.0 cc.	a = 0.5 cc. S = 7.0 cc.	a = 2.0 cc. S = 7.0 cc.	a = 0.5 cc. S = 2.0 cc.
°C.						
15	0.0955	0.3740	0.424	0.2048	0.0802	0.909
16	47	12	22	30	0.0796	05
17	40	0.3684	20	12	90	00
18	33	59	18	0.1998	84	0.896
19	26	34	16	84	79	92
20	19	08	14	70	73	88
21	13	0.3583	12	57	68	84
22	08	58	11	44	63	80
23	02	34	09	31	58	76
24	0.0895	10	07	17	52	73
25	89	0.3488	05	04	48	68
26	83	66	03	0.1891	43	65
27	77	45	02	78	38	61
28	72	24	00	65	34	58
29	68	04	0.398	62	30	54
30	63	0.3384	97	49	25	50
31	58	66	95	36	21	47
32	54	48	93	33	17	44
33	49	29	92	20	13	41
34	44	11	91	07	10	37

a indicate gas volume at which pressure is measured.
S indicates volume of solution from which the CO_2 is extracted in the apparatus.
To calculate grams per liter (= mg. per cc.) each factor is divided by 100.

Before each analysis the chamber is washed once with dilute lactic acid, and once with distilled water as described in the section on general gasometric technique, above.

Direct urea determination in 0.2 cc. of whole blood or plasma

The urease solution and the 0.26 M Na_3PO_4 for the micro analyses are drawn into 2 cc. micro burettes, provided with capillary tips for delivery as shown in figure 52 on p. 344. The burette with Na_3PO_4 is guarded from atmospheric CO_2 by a small soda lime tube at the top. The most frequent source of error in this analysis is negligence in guarding the alkali phosphate from atmospheric CO_2

One cubic centimeter of 0.02 N lactic acid is placed in a small test tube (6 or 7 mm. inner diameter). The 0.2 cc. of blood from a pipette calibrated to *contain* that amount, is run into the lactic acid, and the pipette is rinsed twice by drawing the acid up into it. The blood solution, together with a drop of caprylic-ethyl alcohol, is transferred to the chamber of the blood gas apparatus, 0.6 cc. of water, in portions of 3 or 4 drops each, being used to wash adherent drops of blood solution from the test tube into the cup of the apparatus, and thence into the chamber.

After the 0.2 cc. of blood and the acid have been brought into the chamber, the CO_2 is removed by extracting three times, as described above for 1 cc. samples.

Then 0.07 cc. of the 0.26 M Na_3PO_4 is delivered into the chamber through a mercury seal as shown in figure 52, p. 344. Just before the burette tip is thrust into the mercury in the cup, 2 or 3 drops of the phosphate are wasted, in order to remove the CO_2-containing drop of fluid from the burette tip. After the phosphate, 0.2 cc. of the urease solution is similarly delivered into the chamber, making the total volume of fluid there approximately 2 cc.

The enzyme-phosphate solution is mixed with the blood by lowering the mercury to the bottom of the chamber and permitting it to rise again. The urease is allowed to act on the urea for one minute (or longer if a weaker enzyme necessitates it). Finally the mixture is acidified by admission of 2 or 3 drops of N lactic acid, the CO_2 is extracted by two minutes shaking of the evacuated chamber, and the pressure p_1, is measured, with the gas volume at 0.5 cc. The vacuum is released; 1 or 2 drops of 5 N sodium hydroxide are admitted to absorb the CO_2, the meniscus of the solution is lowered again to the 0.5 cc. mark, and pressure p_2 is read on the manometer.

A blank determination is performed in which the blood is replaced by water. The difference between p_1 and p_2 in the control analysis is designated as c. The pressure of CO_2 from urea is

$$P_{urea} = p_1 - p_2 - c$$

Before each determination the apparatus is washed once with dilute lactic acid and twice with distilled water.

Calculation of results of urea analyses

The factors by which the P_{urea} values obtained in the above analyses are multiplied in order to give the urea contents of urine or blood are given in tables 34, 35, and 36.

The factors were calculated from those in equation 5, page 283 by which millimols of CO_2 per liter are reckoned. Since each mole of urea yields one of CO_2 the factor for mM. CO_2 per liter is converted into one for mg. urea per 100 cc. by multiplying the former by 0.1 the molecular weight of urea. Similarly mg. urea nitrogen per 100 cc. are obtained by multiplying the millimolar CO_2 factors by 0.1 the molecular weight of N_2.

For the Folin-Wu filtrate analyses, it was found that in the mixture extracted—viz., 1 volume of filtrate plus 0.1 volume of urease solution in 50 per cent glycerol plus 0.04 volume of 1 N lactic acid—CO_2 had a solubility only 0.945 times the solubility of CO_2 in water. Hence in calculating the factors for the Folin-Wu filtrate analyses the α' values of table 27 have been multiplied by 0.945. For the other analyses, in which blood or urine was diluted with water containing relatively little salt, the α' values of water were used.

Gasometric standardization of urease activity

In studies on the mode of action of urease (see discussion in chapter XI) it has been shown that the maximum activity of the enzyme is exerted at a pH of approximately 7, maintained by phosphate buffers, in the presence of a high concentration of urea. To determine the activity of urease Van Slyke and Cullen (52) caused the enzyme to act on a solution containing 0.25 M KH_2PO_4, 0.25 M Na_2HPO_4, and 1 M urea concentrations.

The present Squibb's urease, made according to Cullen and Van Slyke's method, but from jack beans instead of soy beans, can decompose at 20° from 0.05 to 0.10 its weight of urea per minute.

The activity is determined in the gas apparatus as follows: 2 cc. of the above phosphate-urea solution of Van Slyke and Cullen (52) at room temperature are run into the chamber of the apparatus. One cubic centimeter of water is placed in the cup, and 0.1 cc. of 5 per cent urease, containing 5 mg. of enzyme, is run underneath the water from a capillary pipette. The enzyme solution, followed by 0.9 cc. of water, is run into the chamber. The time is noted. The intermixture of enzyme and urea solution is quickly completed by lowering the mercury in the chamber a few centimeters and letting it rise again. After an interval, usually five minutes, in which sufficient urea is decomposed to yield 200 to 400 mm. of CO_2 pressure, 0.5 cc. of 1 N lactic acid is run

into the chamber. The acidification stops the action of the enzyme and frees the CO_2 of the ammonium carbonate formed. The CO_2 is extracted, and the manometer reading p_1 is taken with the gas volume at 2.0 cc. The apparatus is washed out twice with water, and a control analysis is run without enzyme, the reading obtained being p_0.

The number of milligrams of urea that 1 mg. of enzyme preparation can decompose at 20° in one minute is found by multiplying $(p_1 - p_0)$ by the factor in table 37 corresponding to the temperature, and dividing

TABLE 37

For Calculating Activity of Urease. Factors by Which Millimeters of Pressure from Urea CO_2 Are Multiplied to Give Milligrams of Urea That Would Be Decomposed at 20° (from Van Slyke (49))

TEMPERATURE	FACTOR	TEMPERATURE	FACTOR
°C.		°C.	
15	0.0103	25	0.0050
16	0.0096	26	47
17	89	27	43
18	85	28	40
19	77	29	37
20	72	30	35
21	68	31	33
22	62	32	30
23	58	33	28
24	54	34	26

by the number of minutes the enzyme acted and the number of milligrams of enzyme present.

$$\text{Milligrams of urea split per minute at } 20^\circ \text{ by 1 mg. urease} = \frac{(p_1 - p_0) \times \text{factor}}{(\text{minutes action}) \times (\text{mg. urease})}$$

Example. In standardizing a lot of Squibb's urease the $p_1 - p_0$ value was 396 mm. at 22.5°, 5 mg. of the enzyme having acted five minutes. Inserting these values into the above formula we obtain:

$$\left.\begin{array}{c}\text{Milligram of urea split per minute}\\ \text{at } 20^\circ \text{ by 1 mg. urease}\end{array}\right\} = \frac{396 \times 0.0060}{5 \times 5} = 0.095$$

Calculation of factors in table 37. Van Slyke and Cullen found that between 10° and 50° the effect of temperature on urease activity is indicated by the equation:

$$\text{Log} \frac{\text{activity at } t_1}{\text{activity at } t_2} = 0.029\,(t_1 - t_2)$$

where t_1 and t_2 are any two temperatures within the above range. If t_1 is 20° and t_2 is t°, the temperature of the analysis, this equation becomes

$$\text{Log} \frac{\text{activity at } 20^\circ}{\text{activity at } t} = 0.029\,(20 - t)$$

which may also be expressed as

$$\frac{\text{Activity at } 20^\circ}{\text{Activity at } t^\circ} = 10^{0.029\,(20 - t)}$$

Hence the factors indicating mg. of urea are each multiplied by $10^{0.029(20-t)}$ in order to correct for the effect of temperature on the activity of the enzyme. The resulting combined factors are those of table 37. They are carried out only to two places, because the velocity determinations performed as described are not sufficiently accurate to justify a third figure.

APPROXIMATE DETERMINATION OF UREA IN BLOOD AND URINE BY THE HYPOBROMITE REACTION. VAN SLYKE (51)

Hypobromite is a much less specific reagent than urease for urea. According to the conditions of the reaction with hypobromite varying proportions of nitrogen gas from other nitrogenous products, such as uric acid and creatinine are evolved.[11] From ammonia the nitrogen is driven off completely as from urea. With urea itself the reaction does not yield quite the theoretical amount of nitrogen gas indicated by the equation

$$CO(NH_2)_2 + 6\ Br + 6\ NaOH = Na_2CO_3 + 6\ H_2O + 6\ NaBr + N_2$$

In the procedures for analysis of urine and blood outlined below, the hypobromite solution used is that previously described for gasometric ammonia determination in micro Kjeldahl analyses. This reagent with pure urea solutions yields up to 99 per cent of the theoretical amount of nitrogen gas, the yield being higher when the hypobromite is more dilute.

For urine analyses we have followed Stehle (41) in first removing ammonia with permutit. When the resultant filtrate is allowed to react with hypobromite for one to two minutes in the manometric apparatus, the amount of non-urea nitrogen evolved approximately compensates for the deficit of N_2 from urea. In urines with relatively low percentage of total nitrogen in the form of urea, the N_2 from the non-urea substances will some-

[11] For a discussion of the hypobromite reaction, see the general chapter XI on urea determination.

what overcompensate for the 5 per cent deficit in the nitrogen gas yield from urea itself; while in urines, such as are obtained with very high protein diets, with relatively high percentages of the total nitrogen in the form of urea, the non-urea substances will fall somewhat short of compensating. A plus or minus error amounting to 4 per cent of the urea present may thus occur. The hypobromite method is not to be used when such an error would invalidate the interpretation of the results.

In blood filtrates the ammonia is negligible, but the relative proportion of other non-urea nitrogenous substances, not so simply removed, is much greater than in urine. Consequently hypobromite with blood filtrates evolves more nitrogen than is contained in the urea present. Under the conditions outlined below for use of the hypobromite reaction with the Folin-Wu filtrate, the yield of N_2 usually indicates in human blood from 1 to 4 mg. more of urea nitrogen per 100 cc. of blood than is present, according to exact analysis with urease. If a correction of 2 mg. is subtracted, the hypobromite nitrogen figure thus corrected falls usually within 1 or 2 mg. of the correct value. Because of the margin of error the hypobromite method is not recommended when the blood urea content is to be compared with the urea excretion rate (blood urea clearance, chapter XI), in order to ascertain whether there is a moderate diminution of renal function. The 2 mg. error which may occur may exceed 20 per cent of the blood urea nitrogen and lead to a corresponding error in the interpretation of the results.

However, the hypobromite blood urea determination is adequate when one wishes only to ascertain whether sufficient urea has been retained to raise the blood urea level above the maximum normal limit. The hypobromite urea nitrogen is the quickest and simplest of all blood nitrogen determinations, duplicate analyses are easy to repeat with constancy extraordinary for micro analyses, and the determination may well replace that of non-protein nitrogen in laboratories where the latter is a routine procedure for detection of nitrogen retention.

Reagents

Hypobromite solution. The same described above on page 356 for manometric micro Kjeldahl analyses.

Permutit. See chapter 12.

Procedure for urine analysis

One cubic centimeter of concentrated urine (sp. gr. over 1.030), or 2 cc. of more dilute urine, are placed in a 100 cc. Erlenmeyer flask, and either 19 or 18 cc. of water from a burette are added, to make the volume

up to 20 cc. Three grams of permutit are added and the mixture is shaken four minutes to remove ammonia. The fluid is then filtered through a dry filter.

One cubic centimeter of water is placed in the cup of the manometric apparatus. Two cubic centimeters of urine filtrate are either layered under the water, or are pipetted through it into the chamber of the apparatus, as shown in figure 29. After the pipette has been withdrawn, the water in the cup is run into the chamber, and is followed by 1 cc. of the hypobromite solution. The cock is sealed with a drop of mercury. The mercury in the chamber is at once lowered to the 50-cc. mark and the chamber is shaken, according to the temperature, for one and one-half minutes at 25°, two minutes at 20°, or three minutes at 15°. The volume of gas is then reduced to 2 cc. and the pressure p_1 is read on the manometer.

A blank analysis is run, in which 2 cc. of water, previously shaken with permutit, replace the urine filtrate. The manometer reading is taken as p_0.

The chamber of the apparatus need not be washed between the successive analyses of a series. Consequently analyses can be run off at the rate of about one every four minutes.

One blank analysis, run at the beginning, serves for an entire series of analyses. If the temperature in the water jacket of the apparatus rises between the time at which the blank was run and the time of the urine analysis, 1.3 mm. are added to p_0 for each degree of temperature increase, to correct for rise in vapor tension; and a similar correction is subtracted from p_0 if the temperature falls.

Calculation of urine urea. The pressure of N_2 is

$$P_{N_2} = p_1 - p_0$$

The urea content of the urine is calculated by multiplying P_{N_2} by a factor from table 39.

Procedure for blood urea

The proteins are precipitated by the usual Folin and Wu tungstic acid method, described on page 65 of chapter II.

Five cubic centimeters of the filtrate, equivalent to 0.5 cc. of blood, are pipetted through a mercury seal into the chamber of the manometric apparatus, in the manner shown in figure 52. One cubic centimeter of hypobromite solution is then passed into the chamber in the same man-

ner. The mercury in the cup is then run down into the chamber, only enough remaining above the cock to fill the capillary. The mercury level in the chamber is lowered to the 50-cc. mark and the chamber is shaken for one and one-half minutes at 25°, two minutes at 20°, or

TABLE 38

HYPOBROMITE METHOD FOR UREA

or urea in the sample analyzed (from Van Slyke (51))

TEMPERATURE	FACTORS GIVING MILLIGRAMS OF UREA N		FACTORS GIVING MILLIGRAMS OF UREA	
	a = 0.5 cc.*	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.
°C.				
15	0.000780	0.003121	0.001671	0.00669
16	77	10	66	67
17	75	0.003099	60	64
18	72	87	54	62
19	69	76	48	60
20	67	65	42	57
21	64	54	36	55
22	61	43	30	53
23	59	33	24	50
24	56	22	19	48
25	53	11	13	46
26	50	01	07	44
27	48	0.002990	01	41
28	45	79	0.001596	39
29	43	69	90	37
30	40	59	85	35
31	37	49	80	32
32	35	39	74	30
33	32	29	69	28
34	30	18	63	26

* a indicates the volume at which gas pressure in the Van Slyke-Neill apparatus was read.

three minutes at 15°. The reaction must not be allowed to continue longer; slow decomposition of non-urea nitrogenous substances would occasion too high results.

The meniscus of the solution is brought to the 0.5-cc. mark in the chamber in analysis of bloods of urea nitrogen content up to 40 or 50 mg.

per 100 cc. For blood with higher urea content the 2.0 cc. mark is used. The reading on the manometer is taken as p_1.

A blank analysis is performed with a 5-cc. portion of 0.9 per cent NaCl solution replacing the blood filtrate. The manometer reading is recorded as p_0. It is taken with the gas volume at both the 0.5 and 2.0-cc. marks, in order that p_0 values shall be available for bloods of either normal or high urea content. The 0.9 per cent NaCl solution has the same solubility for air as the Folin-Wu filtrate. Hence the blank analysis corrects for dissolved air in the sample of filtrate. In order to make the correction exact, the temperature of the filtrate should differ by not more than 0.2° from that of the 0.9 per cent NaCl. It is advisable to put a 10 or 20-cc. portion of the latter into a 100 or 200 cc. flask, similar to those used for receiving the blood filtrate, before beginning the analysis of a series of filtrates. One ascertains that the temperatures of all are alike, and then rotates them about the walls of their flasks for about a minute, to make certain that they are all saturated with air at the same temperature.

As in the urine analyses, the chamber of the gas apparatus need not be washed out between analyses, so that a series can be run off rapidly.

If the temperature rises during the interval between the blank analysis and the analysis of the blood filtrate, 2.0 mm. are added to p_0 for each degree of temperature rise, and a similar correction is subtracted if the temperature falls, when the manometer readings are taken with the gas at 0.5 cc. volume. Of this correction 1.3 mm. are for the change in vapor tension of water in the chamber, and 0.7 mm. for pressure change in the amount of air extracted from the 5 cc. of solution analyzed.

When the manometer readings are taken with the gas at 2.0-cc. volume the correction to p_0 for temperature change after the blank analysis is only 1.5 mm. per degree, the pressure change of the admixed air at the larger volume being only 0.2 mm. per degree of temperature change.

Calculation of blood urea. The pressure P_{N_2} of nitrogen gas is

$$P_{N_2} = p_1 - p_0$$

$$\text{Milligrams of urea nitrogen per 100 cc. blood} = (P_{N_2} \times \text{factor}) - 2.0$$

$$\text{Milligram of urea per 100 cc. blood} = (P_{N_2} \times \text{factor}) - 4.0$$

The values of the factors for different room temperatures are given in table 39. The subtraction of 2 mg. of urea nitrogen, or 4 mg. of urea, per

100 cc. of blood, from the amount indicated by the nitrogen gas evolved, is to correct for the N_2 yielded by non-urea substances of the blood filtrate.

TABLE 39

Hypobromite Urea Factors for Urine and Blood

Factors by which millimeters P_{N_2} are multiplied to give urea nitrogen and urea content (from Van Slyke (51))

Temperature	Factors for urine analyses				Factors for blood analyses*			
	Giving grams of urea N per liter		Giving grams of urea per liter		Giving milligrams of urea N per 100 cc.		Giving milligrams of urea per 100 cc.	
	Sample = 0.1 cc. urine (a = 2.0 cc.)	Sample = 0.2 cc. urine (a = 2.0 cc.)	Sample = 0.1 cc. urine (a = 2.0 cc.)	Sample = 0.2 cc. urine (a = 2.0 cc.)	Sample = 0.5 cc. blood		Sample = 0.5 cc. blood	
					a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.
°C.								
15	0.0312	0.01561	0.0669	0.0335	0.1561	0.624	0.335	1.336
16	11	55	67	34	55	22	34	31
17	10	49	64	32	49	20	32	26
18	09	44	62	31	44	18	31	22
19	08	38	60	30	38	15	30	17
20	07	33	57	29	33	13	29	13
21	05	27	55	28	27	11	28	08
22	04	22	53	27	22	09	27	03
23	03	16	50	25	16	06	25	1.298
24	02	11	48	24	11	04	24	94
25	01	06	46	23	06	02	23	90
26	00	00	44	22	00	00	22	85
27	0.0299	0.01495	41	21	0.1495	0.598	21	80
28	98	90	39	20	90	96	20	76
29	97	85	37	19	85	94	19	72
30	96	80	35	18	80	92	18	67
31	95	74	32	16	74	90	16	62
32	94	69	30	15	69	88	15	58
33	93	64	28	14	64	86	14	54
34	92	59	26	13	59	84	13	50

* To approximate the true urea content of blood, subtract from the blood urea values calculated by the above factors, 2 mg. of urea N or 4 mg. of urea per 100 cc. of blood, as correction for the N_2 yielded by non-urea substances in blood filtrate. No correction is required for urine urea values calculated by the above factors.

The factors in tables 38 and 39 are calculated as described in the following section on amino nitrogen determination, except that the millimols of N_2

gas are multiplied by 28.02 instead of 14.01 to obtain milligrams of nitrogen in the sample. In the factors of tables 38 and 39 no correction is made for the fact that the hypobromite reaction yields only 0.95 mol of N_2 per mol of urea under the conditions of urine analysis, and 0.98 mol under the conditions of blood analysis. The deficit of N_2 yield from the urea is compensated by N_2 from other substances in the urine, and more than compensated in the blood.

AMINO NITROGEN OF AMINO ACIDS AND OTHER PRIMARY ALIPHATIC AMINES. VAN SLYKE (50)

Aliphatic amino nitrogen can be determined by measurement of the N_2 gas evolved by the reaction with nitrous acid.

$$RNH_2 + HNO_2 = ROH + H_2O + N_2$$

A special apparatus for this determination was described in 1911 by Van Slyke (43). The more generally useful manometric apparatus, however, gives equally rapid and accurate amino nitrogen determinations and makes possible micro analyses with smaller amounts of material than even the micro type of the former apparatus. The manometric apparatus permits measurement in a 5-cc. sample to $\pm$0.0001 mg. of amino nitrogen per cubic centimeter, this amount causing a change in the manometer reading of 1.3 mm. One can accordingly perform a gasometric blood amino nitrogen determination directly on 5 cc. of the Folin-Wu tungstic acid blood filtrate, without the concentrating to smaller volume which was formerly a necessary preliminary to analysis.

For discussion of the principle of the reaction and the manner in which varying types of aliphatic amines react with nitrous acid under the conditions employed, the reader is referred to the original paper (43). The most significant facts are that the NH_2 groups in the α-amino acids react quantitatively in three or four minutes at room temperature; while NH_2 groups in other types of substances react much more slowly. Of ammonia about 25 per cent reacts in the time required for complete reaction of α-amino acids (43), and of urea only 6 to 7 per cent (20).

The reaction is carried out by mixing three solutions; viz., of sodium nitrite, acetic acid, and amine. In the manometric apparatus any two of the reagents, AcOH and $NaNO_2$, $NaNO_2$ and RNH_2 (in neutral or alkaline solution), or AcOH and RNH_2, may be mixed and freed of air in the extraction chamber, and the third reagent then added. The last of the above three orders has proved in general to be preferable. The amine solution and acetic acid are mixed and freed of air in the chamber and the $NaNO_2$ is then

added in saturated solution. The saturated nitrite solution need not be freed of dissolved air before it is used. Because of its high salt content (60 grams per 100 cc.), this solution when saturated with air at room temperature dissolves only 0.2 volume per cent of the atmospheric gases (one-tenth as much as water), of which one-third is O_2 and disappears by combination with NO during the reaction. The amount of dissolved atmospheric N_2 carried into the apparatus by the 2 cc. of nitrite solution exerts only 4 mm. of pressure when the gas is measured at 0.5-cc. volume, 1 mm. when at 2 cc. The corrections for these small amounts of dissolved air are too small to vary measurably with room temperature or barometric pressure, and are automatically included in the blank analysis on the reagents.

The entire procedure requires twelve to fifteen minutes. The maximum amount of amino nitrogen that can be determined in a sample is about 0.6 mg., which at 2-cc. volume yields nitrogen gas giving a little over 400 mm. of pressure. The minimum amount measurable in micro determinations is about 0.0004 mg., which yields nitrogen gas giving 1 mm. of pressure at 0.5-cc. volume. Since samples of 5 cc., and, if desired, greater volume can be analyzed, a concentration of 0.01 mg. of amino nitrogen per cubic centimeter suffices for an analysis capable of 1 per cent accuracy.

Reagents

Sodium nitrite solution. Eight hundred grams of $NaNO_2$ dissolved with the aid of warming in 1 liter of water.

Glacial acetic acid.

Alkaline permanganate. Fifty grams of $KMnO_4$ are shaken with 1 liter of 10 per cent NaOH solution until the latter is saturated with the permanganate.

Caprylic alcohol. This is used when necessary to prevent foaming of viscous solutions.

Procedure

The analysis consists of the following steps.

1. The amine solution and acetic acid are freed of air in the apparatus.
2. Sodium nitrite solution is added and the resultant nitrous acid is permitted to react for the necessary time, three to four minutes in the case of α-amino acids at room temperature of 25° to 20°.
3. The mixture of N_2 and NO (the latter formed by spontaneous decomposition of HNO_2) is transferred to a Hempel pipette of the type illustrated in figure 55, where the NO is absorbed by permanganate.

4. The chamber of the manometric aparatus is washed free of nitrous acid and the purified N_2 gas is returned from the Hempel pipette.

5. The amount of N_2 gas is measured by the pressure it exerts at either 0.5 or 2.0-cc. volume.

The details of the successive steps follow:

1. Removal of air from mixed solution of amine and acetic acid. The sample of amine solution may vary from 1 to 8 cc. in volume. Five cubic centimeters is a convenient size. The amine solution is run into the chamber of the manometric apparatus followed by 1 cc. of glacial acetic acid. The most convenient way to add the amine solution is to run it directly into the chamber from a rubber-tipped, stop-cock pipette, as shown in figure 30. However, one may run the solution from an ordinary pipette into the cup of the apparatus and wash into the chamber with small amounts of water, or with the 1 cc. of acetic acid divided into three or four portions. The total volume of fluid added should be known, because the time required for the subsequent reaction is proportional to the dilution of the reagents. If protein or other content of the amine solution makes the latter likely to form troublesome foam, a drop of caprylic alcohol is added with the acetic acid. The amine solution and acetic acid being in the chamber, the cock of the latter is sealed with a drop of mercury, and the chamber is evacuated and shaken for 2 minutes at the usual tempo of 300 to 400 times per minute. The air extracted from the solution is then ejected from the chamber by the technique described on page 279.

Decomposition of amino groups. Two cubic centimeters of the nitrite solution are measured in the cup and run down into the chamber. Evolution of N_2 and NO begins at once. The cock is sealed with a drop of mercury and the chamber is evacuated until the mercury in it has fallen to a level 1 or 2 cm. above the 50-cc. mark. The reaction mixture is permitted to stand in this position until within one minute of the end of the reaction time, given below. During the last minute the mixture is shaken to complete the evolution of the N_2 formed. The relatively large amount of NO gas evolved with the N_2 by spontaneous decomposition of the nitrous acid tends to press the mercury meniscus down into the tube below the chamber. To prevent this, one admits mercury from the leveling bulb once or twice during the shaking, so that the mercury meniscus in the chamber remains within a centimeter of the 50-cc. mark.

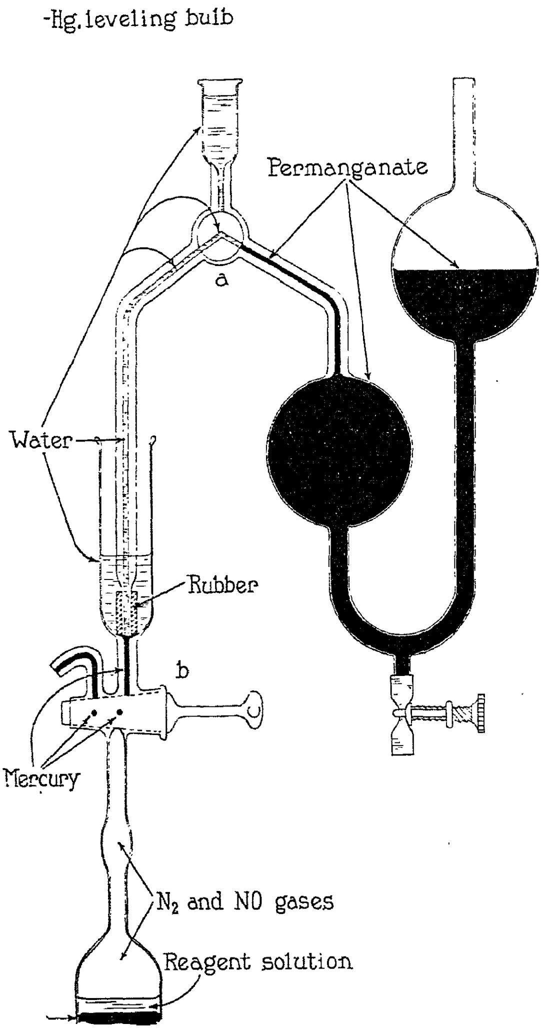


FIG. 55. Apparatus prepared for transfer of N_2 and NO gas mixture to Hempel pipette by turning cock *b*. From Van Slyke (50).

The time required for complete decomposition of the alpha NH_2 groups of amino acids, measured from the moment when the nitrite is run into the chamber till the end of the minute of shaking, varies with the temperature, and at a given temperature it is proportional to the

volume to which the reagents are diluted. When the amine solution plus the water added with it is 5 cc., so that with the acetic acid and nitrite the total volume of solution in the chamber is 8 cc., the time required for quantitative reaction of α-amino acids is three minutes at

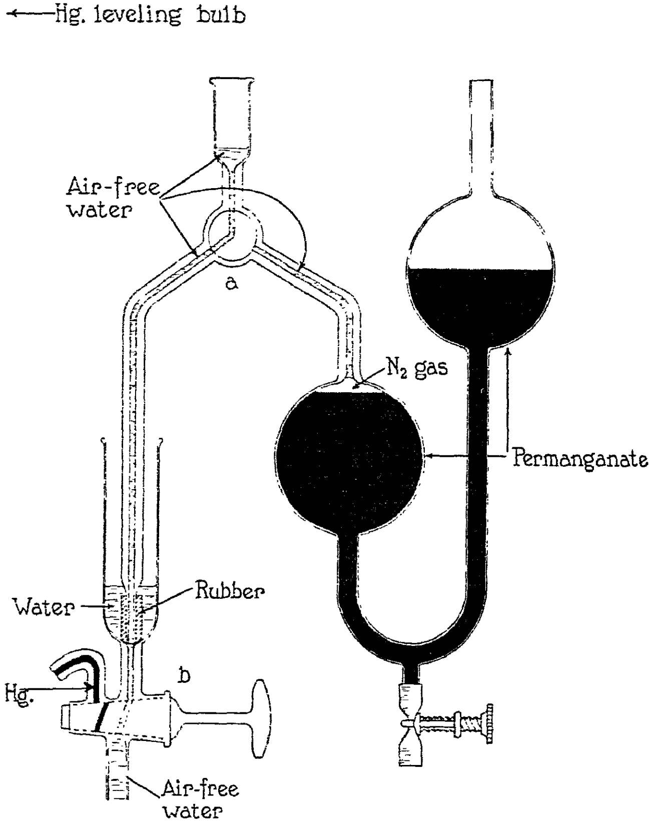


FIG. 56. The NO has been absorbed by permanganate in the Hempel pipette, and the reagents in the chamber have been replaced by air-free water. The Hempel pipette is shown placed again in connection with the chamber, from which air-free water has been forced up into the capillaries and the cup of the pipette. From Van Slyke (50).

25°, four minutes at 20°, and six minutes at 15°, as indicated by figure 58. If the volume of the mixed solutions is greater or less than 8 cc., the reaction time is proportionally increased or diminished.

3. Transfer of NO + N_2 gas mixture to permanganate pipette, and absorption of NO. After the reaction between amine and nitrous acid is completed, the mercury leveling bulb of the apparatus is raised to the high level indicated in figure 55 and the cock (not shown in the figure) connecting the leveling bulb with the gas chamber is opened. The

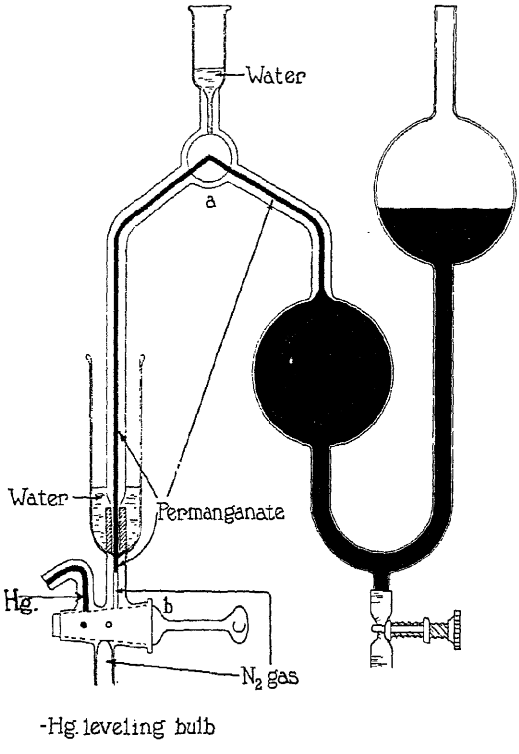


FIG. 57. The mercury leveling bulb has been lowered and N_2 gas drawn back into the chamber, followed by permanganate solution as far as the point indicated in the capillary above the cock of the chamber. From Van Slyke (50).

mercury rises in the chamber, and the gases in it collect at the top under positive pressure, as shown in figure 55. Two or three cc. of water are placed in the cup above the chamber, and the Hempel pipette, with its capillaries filled with water, is pressed firmly into the position shown in figure 55. Cock *a* is turned into the position shown in figure

55. Then by opening cock *b* the gases are forced over into the pipette. When the nitrous acid solution, following the gases, has travelled up the pipette capillary nearly as far as cock *a*, both cocks *b* and *a* are closed. It is preferable not to let any of the nitrous acid solution pass over into the permanganate, because it exhausts the latter unnecessarily. The wasting of a few cubic millimeters of the gas does not affect the results, because the proportion of N_2 in the $N_2 + NO$ mixture is so small that the volume of N_2 lost is negligible.

With cock *a* in a position intermediate between those shown in figures 55 and 56, the Hempel pipette is disconnected from the extraction chamber. The gas in the capillary between cock *a* and the permanganate bulb is forced down into the latter by water from the cup. The remainder of the water in the cup is driven out through the left hand capillary, to wash the nitrous acid solution out of it.

The disconnected Hempel pipette is gently shaken horizontally by hand to absorb the NO gas. The time required is twenty to forty seconds, depending on whether the amount of N_2 approximates the minimum or maximum determinable by the method. The pipette is then set aside. (It may be conveniently suspended from a hook at the right of cock *a*.)

4. Return of purified N_2 gas to manometric apparatus. Before the N_2 is returned to the gas chamber, the nitrous acid solution is ejected from the chamber, which is then washed twice by the following technique. The mercury leveling bulb is placed in its ring at the low level, where it evacuates the chamber. As the mercury in the latter falls, 10 or 15 cc. of water, but no air, are admitted to the chamber from the cup at its top. The bulb is then raised, and the water is ejected. Thirty seconds suffice for each washing.[12] After the second washing 10 cc. of water, measured in two portions from the cup, are admitted into the chamber, which is evacuated and shaken for one minute to remove the greater part of the air from the water. The extracted air is ejected, and 1 cc. of the water is forced up into the cup above the chamber.

The N_2 gas from the Hempel pipette is now returned to the chamber. As shown in figure 56, the capillary between cocks *a* and *b* is filled

[12] In case large amounts of protein are present in the solution analyzed, deaminized protein is precipitated and floccules of it are likely to adhere to the walls of the chamber when the nitrous acid is ejected. Such particles are dissolved by running in a few cubic centimeters of 5 or 10 per cent NaOH solution and raising and lowering the mercury and the alkali solution in the chamber. The latter is then washed twice with water, as above described.

with water from the chamber, and a little water is forced up into the cup above cock *a*, and over into the permanganate bulb. The mercury leveling bulb is now lowered to its middle position, level with the bottom of the extraction chamber, and the N_2 is admitted from the pipette to the chamber. The flow of gas to the chamber can be regulated either by cock *b* or by the cock (not shown in the figures) which connects the chamber to the mercury bulb. The writers prefer the mercury cock because of the nicety with which the flow of mercury, and thereby that of the other fluids, can be regulated. The flow is stopped and cock *b* is closed when the column of permanganate has reached the position shown in figure 57 in the capillary above cock *b*. It is preferable to get as little permanganate into the chamber below as possible, in order to have a clear water meniscus there for reading.

The Hempel pipette is removed, the cup of the gas chamber is washed with water to remove permanganate that may have escaped into it, and about 1 cc. of mercury is run underneath water into the cup. As much of this mercury is run into the chamber as may be necessary to clear of permanganate the bore of cock *b* and the constricted top of the chamber below the cock.

5. *Measuring the nitrogen gas.* The mercury in the chamber is lowered until the water meniscus is at either the 0.5 cc. or the 2.0-cc. mark, according to the amount of gas present. If the gas at 2.0-cc. mark exerts less than 100 mm. pressure it is preferable to use the 0.5-cc. mark, employing a reading glass to locate the meniscus exactly on the line. The manometer reading p_1 is taken. The gas is ejected from the chamber without loss of liquid by the technique previously described, and the manometer reading p_0 is taken with the water meniscus in the gas-free chamber at the same level used for the p_1 reading.

Blank analysis of reagents. A control analysis is carried out in the same manner, except that an equal volume of water replaces the amine solution. The difference $p_1 - p_0$ obtained in the control is the c correction used in the calculation below. For some days or weeks after the sodium nitrite solution has been prepared the c correction appears to diminish and finally to become constant. It is convenient to set aside a considerable amount of nitrite solution and acetic acid on which the c correction has been determined, in order to avoid necessity of frequent redetermination on new solutions. With Merck's "Reagent" nitrite the c correction has been 20 to 30 mm. measured with the gas at 0.5 cc. volume, and one-fourth as much at 2-cc. volume. When there is sufficient amino nitrogen to give over 100 mm. pressure at 2-cc.

volume, variations of the c correction caused by ordinary variation in room temperature may be neglected. For micro analysis, however, with gas measurements at the 0.5-cc. mark the c correction must be determined at a temperature near that of the analysis.

Calculation. The pressure of N_2 gas from the amine analyzed is calculated as

$$P_{N_2} = p_1 - p_0 - c$$

whence the weight of amino nitrogen in the sample is calculated as

$$\text{Milligrams of amino } N = P_{N_2} \times \text{factor.}$$

The values of the factor are found in table 40.

Shortened procedure for series of analyses

When a number of determinations are performed in succession, it is convenient to change the procedure to the following:

The above described p_0 reading is omitted in the amine analysis, the latter being concluded after p_1 is noted. The p_1 reading of the blank analysis is taken as p_0 for the amine analysis. Then

$$P_{N_2} = p_1 - p_0$$

The p_0 thus determined in the blank analysis includes the correction for impurities in the reagents, so that there is no c correction to subtract. The advantage of this procedure is that it obviates one reading with each analysis. The disadvantage is that the p_0 varies with the temperature, due chiefly to effect on vapor tension in the chamber. If the temperature rises between the time of the blank analysis and the subsequent amine analysis, 1.5 mm. may be added to p_0 for each degree rise, subtracted for each degree fall. If the temperature change exceeds 2°, however, it is well to redetermine p_0.

Use of modified Hempel pipette

The details of the structure of the Hempel pipette must meet the requirements stated on page 281.

It is desirable for each day's analyses to fill the pipette with fresh permanganate solution saturated with air at room temperature. Before the solution is put into the pipette, one makes certain that the solution is at room temperature and then whirls 75 cc. of it about the walls of an open

1 liter flask for one or two minutes, in order to bring it into equilibrium with air at atmospheric pressure.

During the course of a series of analyses a film of manganese dioxide forms on the wall of the pipette near the capillary outlet. When the permanganate is renewed the film is as a rule readily detached by shaking water in the pipette. Film which becomes adherent is removed by washing with a saturated solution of oxalic acid in normal sulfuric acid.

Theoretically one would anticipate some error from contact of the pure N_2 gas, left after absorption of NO, with the permanganate solution in the Hempel pipette. The permanganate solution is saturated, not with pure N_2, but with the N_2-O_2 mixture of the atmosphere. The water of the permanganate solution must give off some O_2 to the nitrogen bubble, and absorb some of the N_2. In control analyses it is found, however, that the amount of such exchange which occurs decreases the volume of gas, returned as N_2 to the gas chamber, by only about 0.0012 cc., sufficient to lower the p_1 value 2 mm., when measured at 0.5 cc. volume or 0.5 mm. when measured at 2.0 cc. Error even of this small extent is, however, avoided by using a c correction determined by the same technique, with about the same slight loss of N_2.

Determination of amino acid nitrogen in blood

Of the nitrogenous constitutents of protein-free blood filtrates not only the amino acids, but also the urea reacts measurably with nitrous acid under the conditions of the analysis. In the time used for complete reaction of the α-amino acids about 7 per cent of urea nitrogen is decomposed. In human blood without pathological urea retention the urea nitrogen is ordinarily about twice, at most three times, the amino acid nitrogen content. Under these conditions the amino nitrogen can be determined without preliminary removal of the urea, a correction of 0.07 of the urea nitrogen being subtracted from the total nitrogen obtained by the nitrous acid reaction.

When, however, there is gross urea retention, with blood urea nitrogen above 50 mg. per 100 cc., it is desirable for exact results to remove the urea. The removal is easily accomplished with urease, the resultant ammonia being boiled off before the amino nitrogen is determined.

Accordingly two procedures are described for amino acid nitrogen determination in blood.

Method A. For amino nitrogen in blood of normal or moderately increased urea content

Five cubic centimeters of blood filtrate, prepared by the tungstic acid method of Folin and Wu (see precipitation of proteins, p. 65) and

representing 0.5 cc. of blood, are pipetted into the chamber of the gas apparatus and analyzed as above described. The only difference in detail is that in the present analysis the time of reaction, measured ***from the moment the sodium nitrite solution is run into the chamber to the end of the one minute shaking,*** must be reg-

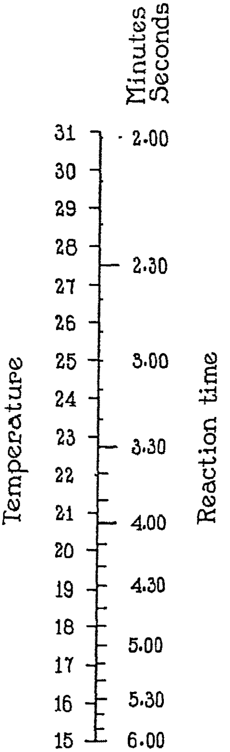


FIG. 58. Scale indicating reaction period for complete decomposition of alpha-amino acids, and 0.07 decomposition of urea, when total volume of reacting solution is 8cc. From Van Slyke (50).

ulated with regard to the temperature somewhat more carefully than is ordinarily necessary, in order that the proportion of urea decomposed shall approximate the constant value of 0.07 allowed for. The reaction periods used for different room temperatures are indicated on the scale of figure 58. With a stop-watch or interval timer one can readily control the reaction time to within ten seconds.

The urea content of the blood is determined independently.

From the amino nitrogen content of the blood calculated by table 40, 0.07 of the urea nitrogen content is subtracted, to correct for N_2 evolved from that proportion of the urea.

Figure 58 is constructed in accordance with the assumption that the speed of reaction between urea and nitrous acid obeys the usual temperature rule of time reactions at room temperature, which double in speed with each 10° temperature increase in accordance with the exponential formula

$$\frac{\theta_2}{\theta_1} = 10^{0.03(t_1 - t_2)}$$

where θ_1 and θ_2 are the periods required at temperatures t_1 and t_2 for the reaction to proceed to a given point. This equation has been found to hold at least approximately for the reaction of nitrous acid with amino acids and urea.

Method B. For amino nitrogen in blood of either normal or high urea content

In this procedure the urea is destroyed with urease. It is desirable to use a relatively small proportion of urease in order to keep down the correction for amino acids in the urease preparation.

The blood sample, 1 to 5 cc., is placed in a flask calibrated to hold the volume of the sample. For each cubic centimeter of blood 1 cc. of a 0.6 per cent KH_2PO_4 solution and 0.02 cc. of a 10 per cent aqueous solution of Squibb's jack bean urease are added. The mixture is permitted to stand an hour at a room temperature of 20° or over.[13]

The proteins are precipitated according to Folin and Wu as described on page 65 with the modification that ***in uremic blood enough extra $\frac{2}{3}$ N sulfuric acid is added to neutralize the ammonia*** formed from the urea. If the blood urea nitrogen is 100 mg. per 100 cc., 0.1 cc. of $\frac{2}{3}$ N sulfuric acid is added per cubic centimeter of blood in addition to the 1.0 cc. ordinarily used. Otherwise some protein

[13] With Squibb's urease, made by the Van Slyke-Cullen (52) method, of the quality at present provided, capable of decomposing per minute nearly 0.1 mg. of urea per mg. of dry urease, 0.02 cc. of 10 per cent urease solution per cubic centimeter of blood suffices, even in uremic cases. If an enzyme preparation is used which, when standardized as previously described in the section on urea determination in this chapter proves to be much weaker, one must either use more or let it act longer. On the other hand half as much enzyme can be used if the digestion time is doubled, or if the temperature is raised to 30°.

may come through into the filtrate. The precipitated blood mixture is brought to 10-fold the volume of the blood and filtered.

To boil off the ammonia formed from the urea, 5, 10, 15, or 20 cc. of filtrate are measured into a Pyrex Erlenmeyer flask of 25 to 150 cc. capacity and a few drops of magnesium hydroxide suspension are added, sufficient to make the entire solution turn white. The mixture is boiled for five or ten minutes in the open flask until the volume has been reduced about one-half. Glacial acetic acid is then added, a drop at a time, until the solution turns acid and the magnesium hydroxide dissolves.[14]

If 10 cc. or more of filtrate have been used the contents of the Erlenmeyer flask are poured into a volumetric flask and the Erlenmeyer flask is washed with small portions of water until the sample has been brought back to its original 10, 15, or 20 cc. volume. Five cubic centimeter portions are used for amino nitrogen determination.

If only 5 cc. of filtrate have been boiled, the Erlenmeyer flask is drained directly into the cup of the gas apparatus, and the volume noted; e.g., 2.7 cc. Then enough water to make this up to 5 cc. is drawn into a graduated pipette and is used in successive portions to wash the flask, whence the washings are poured into the cup of the gas apparatus, and then passed into the chamber.

The *blank analysis* in this case includes some amino nitrogen from the urease, and is performed as follows: 1 cc. of the 10 per cent urease solution is placed in a 10-cc. flask. 0.5 cc. each of 10 per cent sodium tungstate and $\frac{2}{3}$ N sulfuric acid is added, the mixture is diluted to the mark, shaken and, after a half hour's standing to flocculate the proteins, is filtered; 1 cc. of the filtrate is diluted to 50 cc. with water.

The blank analysis is performed with 5 cc. of the diluted filtrate instead of 5 cc. of water.[15]

[14] Boiling with magnesium hydroxide is used to remove ammonia because Osborne, Leavenworth, and Brautlecht (26) found in analysis of hydrolyzed proteins that this treatment removed ammonia quantitatively without appreciably affecting the amino acids. Boiling with more powerful alkalies, even dilute alkali carbonates, splits off ammonia from some of the amino acids. Phillips' milk of magnesia, which by titration is equivalent to an alkali solution of 2.7 N concentration, has proven to be a satisfactory form in which to use this alkali. Magnesium hydroxide is preferable to magnesium oxide because the hydroxide forms an even suspension, and dissolves instantly as soon as an excess of acetic acid is added.

The present method gives with normal blood values for amino nitrogen about 1 mg. per 100 cc. higher than those obtained with the nitrous acid reaction by Bock (7) presumably because Bock used stronger alkali to boil off the ammonia.

[15] The 50-fold dilution is used only when the amount of 10 per cent urease employed has

Calculation. The pressure of N_2 from amino acids in blood is calculated as

$$P_{N_2} = p_1 - p_0 - c$$

where c is the $p_1 - p_0$ value determined in the above blank analysis. P_{N_2} is multiplied by the proper factor in table 40.

With the urease we have used, the c value, measured at 0.5 cc. gas volume, is increased several millimeters by the non-protein urease constituents. The effect could be diminished by using less urease and longer or warmer digestion, or by using especially purified urease, but the correction is sufficiently small and constant to make such refinements appear unnecessary.

The amino nitrogen values by method *B* are usually 0.1 to 0.3 mg. of amino nitrogen per 100 cc. of blood lower than by method *A*. Apparently boiling with $Mg(OH)_2$ decomposes a slight amount of some amino compound in the blood filtrate, although such treatment has been observed to split no ammonia from the amino acids yielded by protein hydrolysis.

Total (free + conjugated) amino acid nitrogen in urine. Van Slyke (43a)

The urea is hydrolyzed with heat and sulfuric acid to ammonia. By treatment with calcium hydroxide the sulfuric acid is precipitated as calcium sulfate and the solution is made alkaline. It is filtered and the filtrate is evaporated to drive off ammonia. In the solution thus freed of urea and ammonia the amino nitrogen is determined as described above.

The amino nitrogen determined is both that of free amino acids and of amino acids combined, as in hippuric acid.

been 0.02 cc. per cubic centimeter of blood. In blood analyses with this amount of urease, each cubic centimeter of blood-urease filtrate contains the non-protein constituents of 0.2 mg. of urease preparation, whereas the undiluted blank filtrate contains per cubic centimeter the non-protein constituents of 50 times as much. In case more or less than 0.02 cc. of 10 per cent urease per cubic centimeter of blood is used, the extent to which the blank filtrate is to be diluted will vary accordingly, being represented by the denominator of the fraction of a cubic centimeter of urease solution used per cubic centimeter of blood.

The reason, in the blank analysis, for precipitating a relatively concentrated urease solution and diluting the filtrate, instead of precipitating an already diluted urease solution, is that the latter would be so extremely dilute that the proteins could not be made to coagulate within a practicable time.

The boiling with magnesium hydroxide has been found unnecessary in the blank, because Squibb's urease is free of ammonia.

Reagents

Reagents for amino nitrogen, as on page 386.
Calcium hydroxide, flocculent finely divided powder.
Concentrated sulfuric acid.

TABLE 40

FACTORS FOR CALCULATION OF AMINO NITROGEN (FROM VAN SLYKE (50))

TEMPERATURE	FACTORS BY WHICH MILLIMETERS OF P_{N_2} ARE MULTIPLIED TO GIVE MILLIGRAMS OF AMINO N IN SAMPLE ANALYZED		FACTORS BY WHICH MILLIMETERS OF P_{N_2} ARE MULTIPLIED TO GIVE MILLIGRAMS OF AMINO N PER 100 CC. BLOOD WHEN FILTRATE SAMPLE IS EQUIVALENT TO 0.5 CC. BLOOD
	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.
°C.			
15	0.000390	0.001561	0.0780
16	389	55	777
17	387	49	774
18	386	44	772
19	385	38	769
20	383	33	766
21	382	27	763
22	380	22	761
23	379	16	758
24	378	11	756
25	376	06	753
26	375	00	750
27	374	0.001495	748
28	372	90	745
29	371	85	743
30	370	80	740
31	368	74	737
32	367	69	734
33	366	64	732
34	365	59	730

Procedure

If the urine contains albumin it is removed with tungstic acid as described on page 535 of the nitrogen chapter. Otherwise the protein would be hydrolyzed by the subsequent heating with sulfuric acid, and the nitrogen of its amino acids would be determined.

25 cc. of urine in a 75 cc. Pyrex test tube are mixed with 1 cc. of con-

centrated sulfuric acid. Each tube is covered with tin foil and placed in a high pressure autoclave. The tubes are heated at 150°, inside temperature of the autoclave, for one-half hour. If the type of autoclave is used which is heated by partial immersion in an oil bath, the temperature of the bath should be about 180°. The hydrolyzed urine is transferred to a 50-cc. flask and 2 grams of the calcium hydroxide are added. The mixture is thoroughly shaken, made up to 50-cc. volume, and filtered through a dry folded filter. Twenty cubic centimeters of the alkaline filtrate are pipetted into a porcelain, or Jena or Pyrex glass, dish of about 50-cc. capacity, and concentrated to dryness on a steam bath. The concentration to dryness requires about a half-hour. The residue is moistened with 2 or 3 cc. of water and 0.5 cc. of glacial acetic acid and is stirred to bring the small amounts of calcium hydroxide and carbonate into solution. The solution is transferred to a 25 cc. volumetric flask and made up to volume with washings from the dish.

Of the solution 5-cc. portions, equivalent to 2 cc. of urine, are used for determination of amino acid nitrogen as described on pages 387–393.

The above method of preparing the urine for the analysis can not be used if glucose is present. It would combine with ammonia during concentration in alkaline solution and form non-volatile amines which would cause the results for amino nitrogen to be too high.

If urine contains glucose it is freed of sugar by treatment with copper sulfate and calcium hydroxide as described in the method for urinary acetone bodies on page 626. The filtrate, containing 10-fold diluted urine, can then be autoclaved and otherwise treated as above described. In this case, since the sample has been so much diluted, it will be desirable, after the filtrate of hydrolyzed urine has been concentrated to dryness, to take 10 cc. instead of 5-cc. portions of the final solution for analysis in the manometric apparatus. Each 10-cc. portion in this case represents 0.4 cc. of urine.

Presumably instead of an autoclave one could hydrolyze the urine in the special pressure tubes devised by Leiboff and Kahn for hydrolysis of the urea in blood filtrates. These are described on page 560 of the urea chapter. Because of the great amounts of CO_2 generated however, it would not be safe to hydrolyze undiluted urine in such tubes. A 3 per cent urea solution would generate something like 15 atmospheres of CO_2 pressure. If the urine were 10-fold diluted before hydrolysis the tubes would presumably stand the pressure.

For *free amino acid nitrogen in urine* see appendix.

MICRO DETERMINATION OF IODATES AND SULFATES, AND ITS APPLICATION TO THE ESTIMATION OF TOTAL BASE IN BLOOD SERUM. VAN SLYKE, HILLER AND BERTHELSEN (58)

Fiske (10) developed for determination of the total base in urine a procedure in which the phosphates were removed by precipitation with ferric hydroxide, and the residue was ignited with sulfuric acid, turning all the bases into sulfates. The SO_4 was then determined by precipitation and titration of benzidine sulfate.

In the gasometric method Fiske's procedure is followed to the point where the SO_4 is determined. Here a different procedure is used whereby the alkali sulfates from 0.16 cc. of serum can be determined as exactly, and somewhat more conveniently, than the sulfates from 1.0 cc. of serum by the benzidine procedure. The gasometric method is based on two reactions. The alkali sulfates are first shaken with an excess of pulverized barium iodate. This is classed as an insoluble salt, but barium sulfate is so much more insoluble that a double decomposition occurs, iodate going into solution and sulfate being precipitated.

$$(1) \qquad Na_2SO_4 + Ba\ (IO_3)_2 \leftrightarrows 2\ NaIO_3 + BaSO_4$$

The mixture is then filtered through a dry filter, and an aliquot of the filtrate is pipetted into the manometric gas apparatus in which an excess of alkaline hydrazine solution has already been placed. An instantaneous and quantitative reaction occurs, according to the reaction

$$(2) \qquad 2\ NaIO_3 + 3\ N_2H_4 = 2\ NaI + 3\ N_2 + 6\ H_2O^*$$

If reaction (1) went completely from left to right, as does reaction (2), 1 mol of SO_4 would yield 3 mols of N_2, and 1 combining equivalent of SO_4 would yield 1.5 mols of N_2. Then SO_4 could be calculated from N_2 simply as:

$$(3) \qquad \text{(Stoichiometric.) Equivalents } SO_4 = \frac{\text{mols } N_2}{1.5} = 0.667 \times \text{mols } N_2.$$

However, reaction (1) does not go completely from left to right. The difference in solubility between $BaSO_4$ and $Ba\ (IO_3)_2$ is not quite great enough to cause a complete reaction. Equilibrium is reached when there is still a significant part of the SO_4 in solution not replaced by IO_3. The actual relationships are given by figure 59 and by empirical Equation 4 with a somewhat larger factor for N_2, and an added term.

$$(4) \qquad \text{(Actual) Equivalents } SO_4 \text{ per liter} = 0.724 \times (\text{mols } N_2 \text{ yielded per liter}) + 1.123$$

* This reaction has been used by Riegler (30) for gasometric determination of iodate.

This empirical linear equation is exact when the reaction between Ba $(IO_3)_2$ and alkali sulfate is carried out within the usual range of room tem-

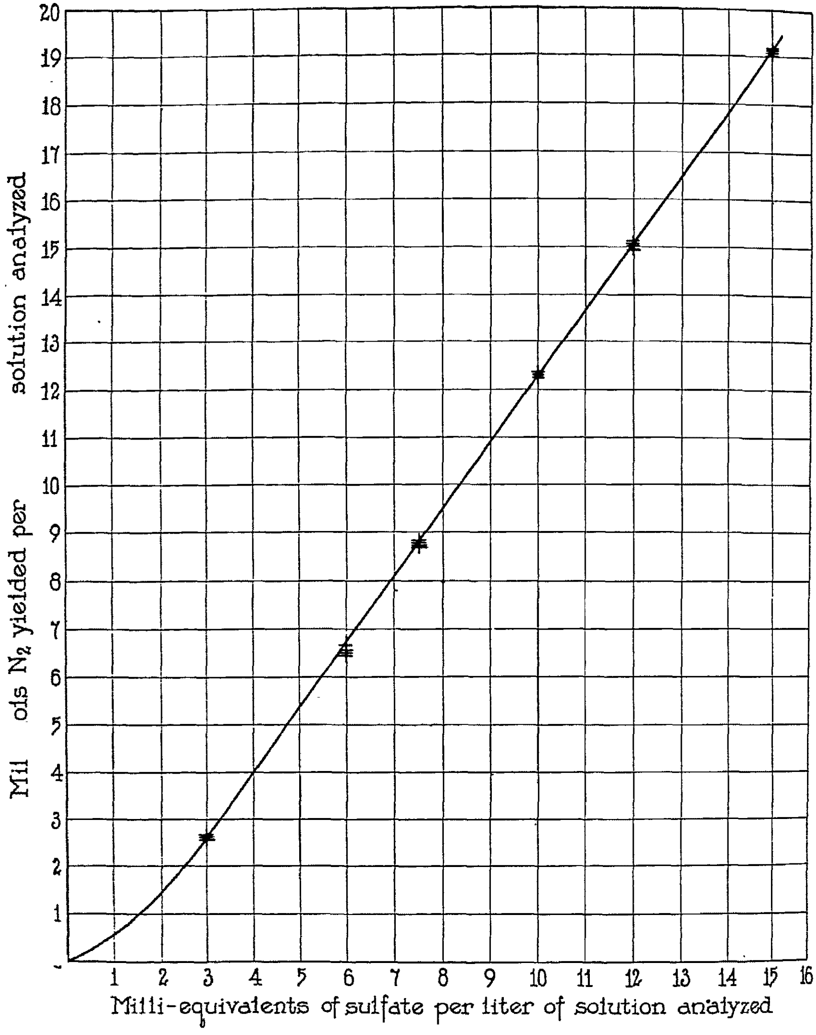


FIG. 59. Relationship between SO_4 present and N_2 liberated by iodate. From Van Slyke, Hiller, and Berthelsen (58).

perature, with SO_4 concentration between 3 and 15 milli-equivalents per liter, pH between 3 and 7, and *in the absence of salts other than sulfates.*

Reagents

Sulfuric acid, concentrated.

Nitric acid, concentrated.

4 N ammonium hydroxide, approximate. Dilute 1 volume of concentrated ammonia solution 4-fold.

1 N sulfuric acid, approximate. Dilute 27 cc. of concentrated sulfuric acid to 1 liter.

Ferric ammonium sulfate. 3.18 grams of ferric alum in 100 cc. of water.

0.1 N ammonium hydroxide, approximate.

Phenol red indicator solution. 0.1 gram of the dry powder is ground in a mortar with 5.7 cc. of 0.05 N sodium hydroxide. When solution is complete, dilute to 250 cc.

Acetic acid solution, 0.2 per cent.

Barium iodate, pulverized (Kahlbaum's has been found satisfactory).

Hydrazine solution. Mix equal volumes of saturated aqueous solution of hydrazine sulfate and 40 per cent sodium hydroxide. The hydroxide solution, 40 grams NaOH per 100 cc., is prepared from purest sodium hydroxide.

Procedure

Ashing with H_2SO_4 and HNO_3. Measure 1 or 0.5 cc. of serum into a large Pyrex or silica test-tube (25 × 200 mm.) which has been previously calibrated at 25 cc. The tube is constricted to 10 or 12 mm. diameter at the calibration mark. Add 0.5 cc. of concentrated sulfuric acid, 1 cc. of concentrated nitric acid and a glass bead. Digest until a dark brown color appears, remove the tube from the flame, and while hot add more nitric acid, a drop at a time, and digest again. Repeat this process two or three times, until the liquid is perfectly clear and all brown fumes have been driven off.

Removal of phosphoric acid. Cool, dilute to about 10 cc. with distilled water, add 1 drop of phenol red. The phosphates are removed as described by Stadie and Ross (see chapter on determination of total base in blood and urine). Neutralize with 4 N ammonium hydroxide, then render just acid with a few drops of normal sulfuric acid. Add 1 cc. of ferric ammonium sulfate solution, and 0.1 N ammonium hydroxide till the full red alkaline color of the indicator develops. Dilute to the 25-cc. mark, and filter through a dry ash-free filter paper.

Ignition of sulfates. Transfer 20 cc. of the filtrate to a silica or Pyrex dish, add 1 drop of concentrated sulfuric acid, and evaporate to dryness on the steam bath. When as dry as possible, heat ***slowly*** on an

electric stove, bringing the stove finally to full heat until all of the sulfuric acid is driven off. Then heat fifteen minutes in the full flame of a triple Bunsen burner. To the residue in the dish add exactly 10 cc. of the acetic acid solution.

Reaction of sulfates with barium iodate. When the residue has gone into solution, pour the solution into a Pyrex test-tube 15 × 2 cm., add 0.25 gram of barium iodate, stopper tightly, and shake *vigorously* for one hour in a shaking machine.

Gasometric determination of dissolved iodate. Filter through a dry ash-free filter paper and estimate the iodate in the filtrate with the manometric gas apparatus. The apparatus, before the first analysis, is rendered gas-free by shaking one minute with about 2 cc. of the hydrazine solution mixed with 2 cc. of distilled water. The gas formed and the solution are expelled from the apparatus, which is now ready for the analysis. (If hypobromite has been used in the apparatus, it will be necessary to clean the chamber and the tube below it with a strong solution of sodium bromide; see p. 413.) Exactly 2 cc. of the hydrazine solution are run into the apparatus through a mercury seal as shown in figure 52. Through the same mercury seal there are then added exactly 2 cc. of the filtrate to be analyzed. The cock of the gas apparatus is sealed with a drop of mercury in the usual way and the chamber is evacuated until the mercury in it falls to the 50-cc. mark. The chamber is then shaken from one to one and one-half minutes. The gas volume is brought to 2 cc.; the pressure on the manometer and the temperature are recorded. The manometer reading is p_1 in the calculation.

Blank. Run through the whole procedure using all reagents and processes described, omitting the serum. The manometer reading in the blank is p_0 in the calculation. Blanks are run with each group of determinations made in the gas apparatus. The blank corrects for impurities in the reagents, for the amount of air dissolved in the hydrazine and iodate solutions (this air is extracted and measured with the N_2 from the iodate), and also for the slight amount of barium iodate which dissolves, by virtue of its slight but measurable solubility, independently of the reaction with the alkali sulfate.

Calculations

The pressure at 2-cc. volume of the N_2 formed by action of iodate on hydrazine is

$$P_{N_2} = p_1 - p_0$$

From P_{N_2} the base content of the serum is calculated by equation 5

(5) Milli-equivalents base per liter serum $= (4.525\,f \times P_{N_2} + 14.0)$

TABLE 41

VALUES OF $0.724\,f$ AND $4.525\,f$ FOR USE IN CALCULATION OF SO_4 AND SERUM TOTAL BASE (VAN SLYKE, HILLER AND BERTHELSEN (58))

TEMPERATURE	f	$0.724f$	$4.525f$
°C			
15	0.1113	0.0806	0.504
16	09	03	02
17	05	00	00
18	01	0.0797	0.498
19	0.1097	94	96
20	93	91	95
21	89	88	93
22	85	86	91
23	81	83	89
24	77	80	87
25	74	78	86
26	70	75	84
27	67	72	83
28	63	70	81
29	59	67	79
30	55	64	77
31	52	62	76
32	48	59	74
33	44	56	72
34	41	54	71

f = factor, from table 30 of this chapter for calculating moles N_2 per liter solution when the gas is extracted from a 1-cc. sample and the pressure is measured with the gas at 2-cc. volume.

If only 0.5 cc. of serum is used as the original sample, the calculation becomes

(6) Milli-equivalents base per liter serum $= 2\,(4.525\,f\,P_{N_2} + 14.0)$

The calculation of SO_4 and total base from observed nitrogen pressures may be facilitated by the use of a table with values of $0.724\,f$ and $4.525\,f$ respectively. These values are accordingly given in table 41.

Example of calculation of total serum base. The value of $p_1 - p_0$ is 280 mm. at 20°, 1 cc. of serum having been ashed. Using the factor 0.495 from the last column of table 41, we obtain

$$\text{Milli-equivalents base per liter of serum} = 0.495 \times 280 + 14 = 138.6 + 14 = 152.6$$

Equations 5 and 6 and the factors in table 41 have been derived as follows. If we call f the factor from table 30 which gives millimoles of N_2 per liter of solution analyzed, when the pressure of N_2 from a 1 cc. sample is measured at 2 cc. volume, then we have, using P in place of P_{N_2},

$$N_2 = \text{millimoles of } N_2 \text{ from 1 liter filtrate} = \frac{P\,f}{cc.\ sample} \tag{7}$$

where P is the pressure difference $p_1 - p_0$ observed in the analysis and "cc. sample" is the cubic centimeters of filtrate (2 cc.) pipetted into the apparatus. We substitute this value of N_2 into equation 4 in order to obtain directly the relationship between the observed manometer pressure and the SO_4 content of the original solution. We thus obtain for the special case in which the sample is 2 cc.,

$$\text{Milli-equivalents of } SO_4 \text{ per liter} = \frac{0.724\,f\,P}{2} + 1.123 = 0.362\,f\,P + 1.123 \tag{8}$$

One liter of the final sulfate solution obtained by the procedure for total base determination described above, when 1 cc. of serum is used, is equivalent to 80 cc. of serum. Hence, for this special case, we multiply the observed SO_4 milli-equivalent concentration of the analyzed final solution by $\frac{1000}{80}$ to obtain the milli-equivalents of total base (combined with the SO_4) per liter of serum. Multiplying the right hand member of equation 8 accordingly by $\frac{1000}{80}$ we obtain equation 5. When only 0.5 cc. of serum is used the factors are doubled, as in equation 6.

REDUCING SUGARS IN BLOOD. VAN SLYKE AND HAWKINS (54)

The methods described below depend on the quantitative reduction of potassium ferricyanide by sugar in an alkaline solution, and the manometric determination of the nitrogen evolved when the excess ferric salt is permitted to react with hydrazine

$$4\ Fe^{+++} + N_2H_4 \rightarrow 4\ Fe^{++} + N_2 + 2\ H_2O$$

The method gives accurately reproducible results, is rapid, requires only simple and stable reagents and no standards. Oxalate and fluoride used as anticoagulants in blood do not interfere. The blood sugar determinations

are made after removal of proteins by the Folin-Wu tungstic acid precipitant, the sulfuric acid, sodium tungstate, and water added to the blood, being for convenience combined into one solution, as described on pages 66–67 of chapter II.

One determines in a control analysis without sugar the pressure p_0, exerted by the N_2 evolved when all the ferricyanide in the reagent reacts with hydrazine in the gas apparatus. The diminished pressure, p_1, is then determined which is exerted by the N_2 evolved from reaction of hydrazine with the amount of ferricyanide left unchanged after part has been reduced to ferrocyanide by the sugar. The pressure difference, $p_0 - p_1$, is proportional to the amount of sugar present. Contrary to most analyses in the manometric apparatus, the zero reading in this case is near the top of the manometer; the more sugar there is in the sample the lower is the manometer reading obtained in its analysis, because the sugar destroys the ferricyanide, of which the N_2 pressure is a measure.

Ferricyanide as a regent for determining reducing sugars has an advantage over copper reagents, in that the ferrocyanide produced by reaction with sugar is not readily reoxidized by the air. The ferricyanide solution partially reduced by heating with sugar may be permitted to stand for several hours at room temperature in contact with air without influencing the results obtained when the analysis is completed by determination of the excess ferric iron. Under the conditions of the analyses described below Van Slyke and Hawkins found that about 7 moles of ferricyanide are reduced by 1 mole of glucose, the exact figure depending somewhat on the conditions of the analysis.

Hagedorn and Jensen (14) first made use of the reduction of potassium ferricyanide for the determination of blood sugar. Ray and Sen (29) have shown that potassium ferricyanide reacts quantitatively with hydrazine in an alkaline solution, liberating free nitrogen gas according to the above equation.

Macro determination of sugar in blood

This determination is performed with a volume of Folin-Wu blood filtrate equivalent to 0.3 cc. of blood. It is called the macro method to differentiate it from the micro method described later, which requires still less material.

Reagents for macro blood sugar determination

Potassium ferricyanide reagent. Eight grams of purest potassium ferricyanide, 75 grams of anhydrous potassium carbonate, and 75 grams of potassium bicarbonate are dissolved in water and made up to 1 liter.

The potassium carbonate and bicarbonate are dissolved in approximately 750 cc. of warm distilled water. The potassium ferricyanide is dissolved in about 100 cc. of distilled water and added to the carbonate-bicarbonate solution. After the mixed solution has cooled to room temperature it is made up to a liter with distilled water and filtered. Filtration is necessary even though the solution appears perfectly clear. The solution is kept in a dark-colored, glass stoppered bottle. It will keep indefinitely in the dark. The reagents do not need to be weighed with any great degree of accuracy, as the control that is run when sugar determinations are made determines the amount of ferricyanide present. (See page 462 for discussion of purification of ferricyanide.)

Alkaline hydrazine solution. A saturated solution of hydrazine sulfate is made by dissolving 25 gm. of the substance in 500 cc. of warmed water and letting the excess crystallize out on cooling.

A 40 per cent solution of the purest grade NaOH is prepared by dissolving 200 grams in water and diluting to 500 cc. Merck's "Reagent sodium hydroxide from sodium" has proven satisfactory. Some other commercial preparations have been found to contain impurities which affect the results.

The alkaline hydrazine solution is made by mixing equal volumes of the saturated hydrazine sulfate and of the 40 per cent sodium hydroxide solution. The mixed solution kept at room temperature has shown no deterioration within three months. How much longer it will keep has not been ascertained.

Procedure for macro blood sugar determinations

Precipitation of blood proteins **is effected by the tungstic acid method with combined Folin-Wu reagents, see pages 66–67.**

Reduction of ferricyanide by blood filtrate. **Three cubic centimeters of filtrate are pipetted into a Pyrex test-tube (14 by 125 mm. outside measure) followed by 1.5 cc. of ferricyanide solution.[16] Both**

[16] It is necessary to use a special 1.5-cc. pipette, with stem of 1-mm. bore, for precise measurement of the ferricyanide. Such pipettes can be obtained from the makers of the manometric apparatus. In case 1.5-cc. pipettes are not at hand, 1 cc. of a ferricyanide-carbonate solution containing, per liter, 12 grams of $K_3Fe(CN)_6$, 100 grams of K_2CO_3, and 100 grams of $KHCO_3$ may be added from a 1-cc. Ostwald pipette with a capillary stem. It is advisable to use the same pipette for the control analysis, in which p_0 is determined, and for the unknown. If this is done the final result is unaffected by any error in the calibration of the pipette. When only 1 cc. of the above 1.2 per cent ferricyanide solution is added, instead of the usual 1.5 cc. of 0.8 per cent ferricyanide, the factors in table 42 for the macro blood method require multiplication by $\frac{4.0}{4.5} = 0.909$, in order to apply, since the 3 cc. of filtrate are diluted only to 4.0 instead of 4.5 cc. with ferricyanide reagent; e.g., the factor for 20° is thus changed from 1.457 to 1.325.

solutions must be measured accurately from Ostwald bulb pipettes with capillary stems. The ferricyanide solution should be added last, in order to facilitate its mixture with the lighter filtrate. The tube is closed by a 1-hole rubber stopper (fig. 60) through which passes a capillary tube 2 cm. long of 0.6 mm. bore. The tube is shaken gently to mix the two solutions, and is then immersed for twenty minutes in a boiling water bath. Usually a series of tubes is heated at once; it is convenient to use a cylindrical copper rack[17] such as is employed by bacteriologists to hold similar tubes. The solutions are cooled by immersing the rack and lower halves of the tubes in cold water for about three minutes and then in water at room temperature.

FIG. 60. Test tube for reduction of ferricyanide by sugar. From Van Slyke and Hawkins (55).

After they reach room temperature the solutions are resaturated with air by shaking them vigorously for one minute. Six or eight tubes are clasped with the fingers so that the tops of the tubes are in the palm of the hand. This insures against the stoppers coming out and also prevents warming the solutions by the heat of the hand. The capillary openings through the stoppers must not be closed. They permit the air, driven out during the heating, to reenter when the tubes are cooled and shaken, while the narrowness of the bore prevents more than an occasional drop of solution from passing out and being lost. The loss of a drop or two does not matter, since an aliquot of the solution is used for the final analysis. After the solutions are thus aerated it is advis-

[17] These copper racks may be obtained from Eimer and Amend, New York, catalogue No. 32002, or from Arthur H. Thomas Company, Philadelphia, catalogue No. 9488.

able to let them stand until analyzed in a beaker of water at room temperature to prevent sudden temperature changes.

It is essential that the resaturation with air shall be complete: the amount of dissolved air in the solution exerts about 60 mm. of pressure in the subsequent manometer reading, and it is essential to have it the same in the control tube and all the filtrate tubes. In practically every case in which analysts to whom we have given this blood sugar method have had trouble with it, the cause has been failure to make the resaturation with air complete. In consequence the solutions in the tubes continue to absorb air slowly as they stand waiting for the gasometric analysis, and those tubes with blood filtrate which stand longer than the control register less fall in N_2 pressure than they should, and therefore yield lower sugar values.

The ferricyanide present in the reagent is enough to oxidize 350 mg. of glucose per 100 cc. of blood. If more is present the filtrate plus ferricyanide solution will, after heating, be entirely colorless, all the yellow ferricyanide having been reduced to colorless ferrocyanide. In such a case another portion of blood filtrate is diluted with an equal volume of water and the analysis is repeated with the diluted filtrate. In calculating the results the factors in table 42 must then be doubled.

Gasometric determination of excess ferricyanide Sufficient alkaline hydrazine solution is run from the cup of the manometric apparatus down into the mercury-filled chamber to reach exactly to the 2-cc. mark on the chamber. The stop-cock of the chamber is closed and the excess hydrazine solution is sucked out of the cup, in which 1 to 2 cc. of mercury are then placed. Through this mercury seal 3 cc. of the previously heated and reaerated sugar-ferricyanide solution are then measured into the chamber of the apparatus from a rubber-tipped stop-cock pipette as described above (see figure 52). The stop-cock is sealed by admitting just enough mercury to fill its bore; mercury drops are not permitted to stream down through the solution in the chamber, as they might reduce an appreciable amount of the ferricyanide solution before it is mixed with the hydrazine. The mercury in the chamber is lowered to the 50-cc. mark. When the ferricyanide and hydrazine solutions mix, their reaction is practically instantaneous, as evidenced by the ebullition of nitrogen gas. The evacuated chamber is shaken rapidly 1 minute to complete the evolution of the gas and the volume of the latter is then reduced to 0.5 cc. (If the chamber is shaken slowly one and one-half minutes may be needed.) The pressure in the manometer is read, p_1, if a blood filtrate is analyzed, p_0 if the determina-

TABLE 42

FACTORS BY WHICH N_2 PRESSURE FALL, p_0-p_1, IN MILLIMETERS IS MULTIPLIED TO CALCULATE REDUCING SUGAR OF BLOOD OR URINE IN TERMS OF GLUCOSE (FROM VAN SLYKE AND HAWKINS (54))*

TEMPERATURE OF GAS CHAMBER	BLOOD ANALYSES. GAS PRESSURES MEASURED AT 0.5 CC. VOLUME		URINE ANALYSES. GAS PRESSURES MEASURED AT 2.0 CC. VOLUME	
	Factors to calculate milligrams of sugar per 100 cc. blood		Factors to calculate grams of sugar per liter urine	
	Macro blood* method	Micro blood method	Urine diluted 1:20	Urine diluted 1:50
°C.				
10	1.508	4.37	0.136	0.340
11	03	5	5	0.338
12	1.498	4	5	7
13	93	2	4	6
14	87	1	4	5
15	82	4.29	4	4
16	77	8	3	2
17	72	7	3	1
18	67	5	2	0
19	62	3	2	0.329
20	57	2	2	8
21	52	1	1	7
22	47	4.19	1	6
23	42	8	0	5
24	37	6	0	4
25	32	5	0.129	2
26	28	4	9	1
27	23	2	9	0
28	18	1	8	0.319
29	14	4.09	8	8
30	09	8	7	7
31	04	7	6	6
32	00	5	6	5
33	1.395	4	6	4
34	91	3	5	3

* In case a blood filtrate, because of great sugar content, is diluted twice as much as prescribed in the directions for usual analyses, the value of the factor F in the table is doubled. To calculate blood sugar in grams per liter (= mg. per cc.) the factors for blood analyses in the table are divided by 100.

tion is that of the control without sugar. The solution is then ejected from the chamber and the apparatus is ready for the next determination.

Preliminary determination of p_0. The control determination is performed with 0.9 per cent NaCl solution in place of the blood filtrate, because this solution has the same solvent power for air as the Folin-Wu filtrate. The dissolved air provides part of the pressure observed in the analysis, and hence must be equal in determination of p_0 and p_1. In all details, heating, aeration, etc., the control determination is carried through exactly like the analysis of the blood filtrate, described below. One control provides the p_0 for an entire series of blood analyses.

TABLE 43

CORRECTION TO p_0 FOR 1° TEMPERATURE CHANGE* (FROM VAN SLYKE AND HAWKINS (54)) FOR USE WITH CALCULATIONS BY TABLE 42

TEMPERATURE RANGE	INCREASE OF VAPOR TENSION OF WATER PER 1° TEMPERATURE RISE	MACRO BLOOD ANALYSES		MICRO BLOOD ANALYSES		URINE ANALYSES	
		Increase of N_2 pressure of control analysis per 1° temperature rise	Total p_0 correction per 1° temperature change	Increase of N_2 pressure of control per 1° temperature rise	Total p_0 correction per 1° temperature change	Increase of N_2 pressure of control per 1° temperature rise	Total p_0 correction per 1° temperature rise
°C.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
15–20	0.7	1.0	1.7	0.5	1.2	1.1	1.8
20–25	1.2	1.0	2.2	0.5	1.7	1.1	2.3
25–30	1.6	1.0	2.6	0.5	2.1	1.1	2.7

* Add correction to p_0 if temperature, observed at p_1 reading, is higher than that at the p_0 reading; subtract correction if temperature at p_1 reading is lower than at p_0 reading. The "total p_0 correction" in each case is the sum of the rise in vapor tension given in the second column plus the increase in N_2 pressure of the control per 1° temperature rise.

Calculation of results of macro blood analysis

The $p_0 - p_1$ reading multiplied by the proper factor from table 42 gives directly the blood sugar in milligrams per 100 cc.

If the temperature, observed on the thermometer in the water jacket of the extraction chamber, has changed at the time of the p_1 reading from the temperature at the p_0 determination, the p_0 value used in the calculation is corrected as indicated by table 43.

Remarks on gasometric technique. It is not necessary to wash the chamber of the gas apparatus between the successive determinations of a series. A great excess of alkaline hydrazine solution is used for each determination and effectively cleans the chamber for the next. The gasometric determinations in a series can be run off at the rate of one every three minutes.

It is necessary that the hydrazine and ferricyanide-sugar solutions be run into the chamber of the gas apparatus in the order directed, the hydrazine first, then the ferricyanide-sugar solution. When thus added, the two solutions divide into layers, with the heavier alkaline hydrazine solution remaining on the bottom in contact with the mercury. The ferricyanide is completely reduced by the hydrazine before coming into contact with the mercury. If the ferricyanide were added first, contact with the mercury in the chamber would partially reduce it before it mixed with the hydrazine.

Before a series of determinations is started it is advisable to run 2 or 3 cc. of the alkaline hydrazine solution into the chamber of the gas apparatus and evacuate and shake the latter for two or three minutes, in order to make sure that there are no impurities present which can oxidize hydrazine.

This treatment has sufficed to prepare the apparatus after any previous use except micro-Kjeldahl or urea analyses by the hypobromite method. The mercuric bromide which apparently clings to the walls of the chamber, or to the rubber connecting tube below it, reacts with hydrazine for a long time. This bromide is readily dissolved by a strong solution (saturated to half saturated) of sodium bromide. Ten or 15 cc. of the latter are run into the chamber of the gas apparatus, and the mercury is lowered until the solution appears in the glass tube below the flexible rubber joint underneath the chamber. If the apparatus has been used for many analyses with hypobromite, it may be necessary to let the sodium bromide solution stand thus in it for 2 hours. The chamber is then washed with distilled water and with alkaline hydrazine solution.

Scale for direct reading of sugar values

Calculation can be obviated by preparing on a strip of millimeter paper a scale on which, when it is fastened beside the manometer tube, sugar percentages can be read off directly from the level of the mercury column at the time of p_1 readings. Such a scale is theoretically exact only for one temperature. However, if two scales are prepared, one for 20° and one for 25°, they can be used to cover the temperature range from 17.5° to 27.5°, with a maximum error of less than 1 per cent.

If, for example, a scale is prepared for the macro blood sugar method at 20°, an interval indicating 10 mg. per cent of blood sugar corresponds to $\frac{10}{1.457} = 6.86$ mm. (see table 42). The scale is made for sugar values up to 350 mg. per cent by marking off 35 intervals, at 6.9, 13.7, 20.6 . . . ., etc. mm. from the top, and marking the points 10, 20, 30 . . . . etc., showing the milligrams per cent of blood sugar indicated by each point. Each 10 mg. interval is divided into five subdivisions indicating differences of 2 mg. per

cent of sugar. The scale is attached by a piece of adhesive tape or other temporary fastening device to the board beside the manometer tube. The zero point at the top of the scale is placed level with the p_0 reading found on the manometer. Then, when a sugar-ferricyanide solution is analyzed, the reading on the scale opposite the mercury meniscus indicates at once the milligrams per cent of blood sugar.

Micro method for sugar determination in 0.2-cc. samples of blood

Reagents

Ferricyanide solution. This contains, like the above ferricyanide, 75 grams each of K_2CO_3 and $KHCO_3$, but only 4 grams of $K_3Fe(CN)_6$, per liter.

Alkaline hydrazine solution. Same as above.

Tungstic acid solution. Two volumes of the combined solution of sodium tungstate, sulfuric acid and water, for the precipitation of blood proteins (see p. 66–67) are diluted with 3 volumes of distilled water.

Procedure for micro blood sugar analyses

Measuring blood sample and removing proteins. When samples of capillary blood are to be analyzed by the micro method, a sufficient number of rubber stoppered centrifuge tubes is prepared, each containing 5 cc. of the dilute tungstic acid precipitating reagent described above. The blood drops forming on the incised finger or ear lobe are drawn by capillary attraction into a dry capillary pipette calibrated to ***contain*** 0.200 cc. The pipette is at once emptied into one of the test-tubes and is rinsed twice by drawing the tungstic acid up into it. The test-tube is then stoppered and shaken. After any convenient interval the tube is centrifuged for five minutes. The 0.200 cc. pipette is made from a capillary tube of about 1-mm. bore, and is calibrated to contain 0.200 cc. by weighing 2.69 grams of mercury in the dry pipette (see "calibration with mercury" and figure 4, chapter I).

Analysis of blood filtrate. The tip of a simple blood pipette, calibrated to ***deliver*** 3 cc., is covered with absorbent cotton to serve as a filter, a point of technique introduced by Somogyi (36). The cover is prepared by placing the tip of the pipette on a small, thin square of absorbent cotton held in the fingers of one hand, and twisting the pipette until the cotton is wound tightly about the tip. Through this filter the pipette is filled with supernatant solution from the centrifuge tube. The filter is then removed from the pipette tip and 3 cc. of filtrate are delivered into a Pyrex glass test-tube (14 × 125 mm.). 1.5 cc. of the ferricyanide reagent are then added.

A control tube is set up containing 1.5 cc. of the ferricyanide reagent and 3.0 cc. of a 0.4 per cent solution of sodium chloride.

The procedure from this point is exactly the same as in the macro method described above.

Determination of sugar in blood containing ether

The blood from an anesthetized subject may contain enough ether to give a vapor pressure of 40 to 50 mm. when the Folin-Wu filtrate, after reaction with ferricyanide, is shaken in the Van Slyke-Neill apparatus. The effect is to make the sugar results come out too low; i.e., p_1 is too high on account of the ether, hence $p_0 - p_1$, which serves as a measure of the sugar, is made too small.

The error is prevented as follows: A mark is etched to indicate 3 cc. content on the small test-tube used for the reaction of ferricyanide with blood filtrate. After the 3 cc. of filtrate have been placed in the tube, the filtrate is heated with a micro burner and is boiled for two minutes to drive off the ether. The filtrate is then cooled and is diluted back to the 3-cc. mark.

Or if more convenient, the tube may be weighed to within 0.01 gram before the boiling; the water which has been driven off is then replaced by weight.

After the ether has been driven off and the water replaced, the tube is cooled to room temperature, ferricyanide reagent is added, and the analysis is continued as usual.

FERMENTABLE SUGAR IN BLOOD BY YEAST METHOD. VAN SLYKE AND HAWKINS (55)

Removal of blood protains. The blood proteins are removed with the modification of Folin and Wu's tungstic acid procedure described in the preceding section on manometric blood sugar determination.

Removal of fermentable sugar from blood filtrate. From one portion of the filtrate, conveniently about 10 cc., the fermentable sugar is removed according to Somogyi. Van Slyke and Hawkins applied the procedure as follows (see also general chapter on sugar determinations):

A portion of Fleischmann's yeast cake is pulverized and suspended in 4 times its weight of water. Of the suspension a volume, approximately equal to that of the blood filtrate sample to be fermented, is placed in a centrifuge tube and washed five times by repeated centrifugation and decantation. After the last centrifugation the water is decanted as completely as possible, and the water film adherent to the

walls of the tube above the packed cells is removed with a roll of filter paper. Adherent water remaining between the cells is not sufficient to dilute significantly the blood filtrate. The blood filtrate is added to the yeast packed in the centrifuge tube. Filtrate and yeast are mixed and permitted to stand at room temperature for fifteen minutes. The mixture is then centrifuged.

Determination of total and unfermentable blood sugar. Portions of 3 cubic centimeters of the supernatant fluid obtained by the yeast treatment are analyzed as described above for macro blood sugar determinations. Similar sugar determinations are made on 3 cc. portions of the untreated blood filtrate.

The p_0 value, for the analyses of the yeast-treated portions, is determined by blank analysis of the supernatant fluid obtained from a centrifuged mixture of 1 volume of washed yeast cells and 4 volumes of water.

It is essential in determining this blank to treat the yeast with water rather than with tungstic acid solution. When water is mixed with washed yeast it extracts the same minimal amount, if any, of reducing material from the cells that a sample of glucose-free (previously fermented) Folin-Wu blood filtrate extracts from them. Hence the water extract gives the correct blank. If yeast is mixed with tungstic acid solution, the latter extracts measurably more reducing material, enough to be equivalent to 5 or 10 mg. per cent of blood sugar, sometimes more. Presumably the reason for this phenomenon is that the fluid in the tungstic acid-yeast mixture has a much greater acidity and contains more titratable acid than filtrate from tungstic acid-blood mixture; in the latter case the blood proteins remove the acid almost completely. The effect of using a tungstic acid extract of yeast cells for the blank determination would be to lower erroneously the p_0 value, and hence to lower the value, calculated as $(p_0 - p_1) \times$ factor, obtained for non-fermentable reducing material in blood.

Calculation of fermentable blood sugar[18]

For calculating both total sugar from analysis of the untreated filtrate and unfermentable sugar from analysis of the yeast treated filtrate, the $p_0 - p_1$ values are multiplied by the usual calculation factors in table 42 for the macro method.

If a temperature change occurs during the interval between the p_0 and p_1 observations, p_0 is to be corrected as indicated in table 43.

[18] If *only* fermentable sugar is desired, only the p_1 values of the two analyses need be determined. Fermentable sugar is then calculated as $(p_{1T} - p_{1N}) \times$ factor, where p_{1T} is the p_1 reading for the total sugar determination, p_{1N} is the p_1 for the non-fermentable sugar analysis, and the factor is from table 42. This procedure eliminates the two p_0 determinations. It can be used, however, only if the yeast employed has been washed quite free of reducing substances.

From the total and unfermentable reducing material the fermentable sugar is found by difference.

Fermentable sugar = (total sugar) − (unfermentable sugar)

FERMENTABLE SUGAR IN BLOOD FROM DECREASE IN REDUCING POWER CAUSED BY SPONTANEOUS GLYCOLYSIS. VAN SLYKE AND HAWKINS (55)

Spontaneous disappearance of reducing sugar from blood was a phenomenon known to Claude Bernard and studied by many later investigators (see Tolstoi (42)). The sugar is transformed into lactic acid, as shown by Evans. Hiller, Linder and Van Slyke (19) and Folin and Svedberg (11) have found that the same amount of reducing substance is removed by spontaneous glycolysis at 38° for twenty or more hours that is removable by short fermentation with yeast.

Procedure

Two analyses, designated as *A* and *B* are required:

***A.* In one sample of fresh blood the total reducing material is determined at once.**

***B.* Another sample of the whole blood in a stoppered tube is incubated at 38° for twenty to twenty-four hours, and the non-glucose reducing material left in it is then determined.**

Calculation. A − B = fermentable sugar.

REDUCING SUGAR IN URINE

The procedure here outlined is designed for use with urines, such as those encountered in diabetes, in which the significant variations in glycosuria are gross enough to be satisfactorily shown by measurement of the total reducing substances. The method is sensitive to 0.02 per cent of glucose in solution. Normal urine may show reducing power equal to that of a 0.2 per cent glucose solution, sufficient to cause a 15-mm. fall in the manometer reading, but due almost entirely to non-fermentable urinary substances which have no apparent relationship to carbohydrate metabolism (see chapter on corbohydrates in volume I).

Reagents for urine sugar

Ferricyanide solution. Twenty-eight grams of potassium ferricyanide and 75 grams each of potassium carbonate and potassium bicarbonate are dissolved in water, made up to 1 liter, and filtered. The solution is kept in a stoppered bottle of dark glass.

Alkaline hydrazine solution. Same as for blood sugar.

Procedure for urine sugar

Dilution of urine. One cubic centimeter of urine is diluted with water ordinarily to 20 cc. In urine so diluted the ferricyanide reagent used will determine up to 3.5 per cent of glucose. In case the sugar content is known to be very high, 1 cc. of urine is diluted to 50 cc., so that glucose up to about 9 per cent can be determined. Dilution is the only preliminary treatment of the urine required. Even albumin does not affect the determination significantly.

Reduction of ferricyanide by diluted urine. Two cubic centimeters of the diluted urine are measured into a Pyrex glass test-tube (14 $\times$ 125 mm.) and 2 cc. of the ferricyanide solution are added. The solutions are mixed, heated twenty minutes, cooled, and aerated, all as described above for blood analyses. A slight flocculent, permanent precipitate, presumably of calcium and magnesium carbonate, appears when the urine filtrate and ferricyanide reagent are mixed, but it does not interfere in any way with the analysis.

Gasometric determination of excess ferricyanide. One drop of caprylic alcohol to prevent foaming is run into the chamber of the manometric apparatus, followed by 2 cc. of alkaline hydrazine solution, measured through a mercury seal as described above on page 410 for blood sugar analyses. Three cubic centimeters of urine-ferricyanide mixture are added through a mercury seal in the same way, and the chamber of the apparatus is shaken for one minute to complete the evolution of the nitrogen gas. The gas volume is reduced to 2.0 cc. and the pressure is read on the manometer, p_1 if urine filtrate is analyzed, p_0 if the determination is a blank. The blank analysis is performed with water in place of urine. The air dissolved in the reagents exerts about 13 mm. pressure; the N_2 from all the ferricyanide in the control exerts about 300 mm.

The "Remarks on Gasometric Technique" made above in connection with the blood sugar determination apply also to the urine analysis.

Calculation consists in multiplying the observed $p_0 - p_1$ value by the factor in table 42, p_0 being corrected by table 43 if temperature changes intervene between the p_0 and p_1 readings. A scale, as described in connection with the blood analysis, may be used to obviate calculation.

FERMENTABLE SUGAR IN URINE BY YEAST. VAN SLYKE AND HAWKINS (55)

Fermentable sugar can be determined in urine by letting yeast act and measuring either the CO_2 that is formed or the decrease in reducing sugar.

Van Slyke and Hawkins (55) applied both methods. Only that based on measurement of decrease in reducing sugars will be given here, however, as it proved to be more accurate than measurement of the CO_2 formed.

The procedure outlined below, with minimum dilution of the urine, is designed primarily for urine with amounts of reducing substances of the order of magnitude found in non-diabetic cases. It is usually in urines of slight reducing power that one needs to determine the fermentability of the material, either for diagnostic or experimental purposes. The method as given is designed for urines with reducing powers not exceeding that of a 0.5 per cent glucose solution.

To determine the fermentable sugar in urines more heavily loaded with reducing substances, such urines are diluted sufficiently to bring the total reducing power below that of a 0.5 per cent glucose solution.

Reagents

Ferricyanide solution. This contains 14 grams of $K_3Fe(CN)_6$, 75 grams of K_2CO_3, and 75 grams of $KHCO_3$ per liter. It is identical with the reagent described for total urine sugar, except that here only half as much ferricyanide is used because of the smaller amounts of reducing material encountered. The solution is to be prepared in the manner directed in the method for total urine sugar above.

Oxalic acid. 0.1 N solution.

Hydrazine solution. Same as that used for total sugar.

Lloyd's reagent. The preparation of Fullers' earth known by this name.

Procedure

Preparation of urine for analysis. Creatinine, uric acid, and other non-glucose materials exert reducing effects which combined usually exceed that of the fermentable sugar in non-diabetic urine. The amount of such substances present is diminished by treatment of the urine with Lloyd's reagent, as described by Folin and Berglund. The substitution of oxalic acid for the sulfuric acid used by them obviates formation of a calcium salt precipitate when the urine filtrate is later mixed with ferricyanide-carbonate solution.

To 10 cc. of urine add 5 cc. of 0.1 N oxalic acid, 5 cc. of water, and 1.5 grams of Lloyd's reagent. Shake gently for two minutes and filter.

A control filtrate for blank analysis is also made at the same time: Add 5 cc. of 0.1 N oxalic and 1.5 grams of Lloyd's reagent to 15 cc. of water, shake for two minutes and filter.

Determination of total reducing material in urine filtrate.

Two cubic centimeter portions of the filtrate are mixed with 2-cc. portions of the ferricyanide reagent in test-tubes, and the analysis is carried

TABLE 44

For Fermentable Sugar in Urine

Factors by which N_2 pressure fall, $p_0 - p_1$ in millimeters, is multiplied to calculate reducing sugar of urine in terms of grams glucose per liter (from Van Slyke and Hawkins (55))

TEMPERATURE OF GAS CHAMBER	FACTOR*	TEMPERATURE OF GAS CHAMBER	FACTOR*
°C		°C.	
15	0.0143	25	0.0138
16	2	26	8
17	2	27	7
18	1	28	7
19	1	29	6
20	0	30	6
21	0	31	5
22	0.0139	32	5
23	9	33	4
24	8	34	4

* These factors hold when, as in the procedure described, undiluted urine is used, and the final 3 cc. of ferricyanide-urine mixture used for the gasometric determination represent 0.75 cc. of urine. If, by reason of high sugar content, the urine is diluted before analysis, the above factors are multiplied as many times as the urine is diluted.

TABLE 45

Correction to p_0 for 1° Temperature Change for Use With Calculations by Table 44

TEMPERATURE RANGE	INCREASE OF VAPOR TENSION OF WATER PER 1° TEMPERATURE RISE	INCREASE OF N_2 PRESSURE OF CONTROL PER 1° TEMPERATURE RISE	TOTAL p_0 CORRECTION PER 1° TEMPERATURE RISE*
°C.	*mm.*	*mm.*	
15–20	0.7	0.6	1.3
20–25	1.2	0.6	1.8
25–30	1.6	0.6	2.2

* Add correction to p_0 when temperature is higher at p_1 reading; subtract the correction when temperature is lower.

out as described above for total urine sugar, in all respects except the difference in ferricyanide reagent. The p_0 value is determined by like analysis of the control filtrate.

Determination of non-fermentable reducing material in urine filtrate. A second portion of urine is treated as follows: To 10 cc. of urine are added 7.5 cc. of a 40 per cent yeast suspension (20 grams of a Fleischmann's compressed yeast cake in 50 cc. of water. The mixture contains, as shown by centrifuging, about 40 per cent by volume of moist yeast). The mixture is allowed to stand for fifteen minutes, then 5 cc. of 0.1 N oxalic acid and 1.5 grams of Lloyd's reagent are added. The mixture is shaken for two minutes and filtered. Under these conditions, the yeast will remove glucose in amounts up to 0.5 per cent in the original urine.

Portions of 2 cubic centimeters of the filtrate are analyzed as in the determination of the total reducing material. The p_0 value is determined by similar analysis of filtrate from a control suspension of yeast and Lloyd's reagent in which water replaces the urine.

The proportions of yeast and fluid are designed to give the same concentration of non-fermentable reducing material in the fluid that is obtained, in the preceding total reducing material determination, by diluting the 10 cc. of urine to 20 cc. with water solutions. In the present yeast-fluid mixture, 10 cc. of urine are mixed with 9.5 cc. of water solution and 3 cc. of yeast cells. The latter were found by Van Slyke and Hawkins to take up about as much non-fermentable reducing material as would the 0.5 cc. of water required to make the fluid volume up to 20 cc.

Calculation of fermentable urine sugar. The results of the two analyses are calculated by means of the factors in table 44. If temperature change occurs between p_0 and p_1 readings, p_0 is corrected according to table 45. The fermentable sugar is calculated as

Fermentable sugar = (total sugar) − (non-fermentable sugar).

CALCIUM IN BLOOD. VAN SLYKE AND SENDROY (63)

The reaction between oxalic acid and permanganate,

$$5\ H_2C_2O_4 + 2\ KMnO_4 + 3\ H_2SO_4 = K_2SO_4 + 2\ MnSO_4 + 8\ H_2O + 10\ CO_2$$

is the basis of the gasometric calcium determination. The calcium is precipitated as calcium oxalate, the precipitate is redissolved in dilute sulfuric acid, and the solution thus obtained is shaken with an excess of permanganate in the manometric apparatus. The CO_2 evolved serves as a measure of calcium.

The procedure is particularly advantageous for micro analyses. The 0.1

mg. of Ca present in 1 cc. of ordinary blood serum yields enough CO_2 to exert at 0.5 cc. volume a pressure of about 160 mm. to read on the Van Slyke-Neill manometer, with an accuracy of 1 per cent.

Reagents

The 20 per cent trichloroacetic acid, 20 per cent sodium acetate, 0.016 per cent brom cresol green, 3.5 per cent ammonium oxalate, 1:1 and 1:50 ammonia water, and *approximately 1 N sulfuric acid* described for the micro titration of blood calcium in the calcium chapter.

In addition to these, the following:

Approximately 0.15 N potassium permanganate solution. 4.8 grams of potassium permanganate are dissolved and diluted to a liter. A portion is acidified before use by the addition of 1/20 volume of 1 N sulfuric acid.

5 N sodium hydroxide, approximate.

Reagents, water, and filter paper used up to the point in the analysis where the precipitation and washing of the calcium oxalate are finished should be tested for calcium as an impurity.

Procedure

Removal of proteins. The proteins of plasma or serum are precipitated, as described in the calcium chapter for microtitration of blood calcium, by mixing with 3 volumes of water followed by 1 volume of 20 per cent trichloroacetic acid. However, when only 1 or 0.5 cc. of serum is available, it is precipitated in a 10 cc. flask with 2 cc. of 20 per cent trichloroacetic acid. The filtrate is caught in an accurately graduated 15-cc. centrifuge tube, the volume is noted, and the entire filtrate is used for analysis.

Precipitation and washing of the calcium oxalate are carried out as described in the calcium chapter, for the microtitration of blood calcium.

Resolution of precipitate and transfer to chamber of manometric apparatus Two cubic centimeters of 1 N sulfuric acid are run down the wall of the centrifuge tube in such a manner that every portion of the wall is washed. The tube is dipped into hot water to acceleratere solution of the crystals, then cooled to room temperature.

The outside rim of the centrifuge tube is smeared with a thin film of vaseline to prevent the solution from creeping over the rim when decanted. The tube is then emptied smoothly, without splashing, into the cup of the Van Slyke-Neill chamber, and the solution is drawn down into the chamber. Four cubic centimeters of water are then used, in

three portions, to wash the walls of the centrifuge tube and the cup of the manometric chamber. The washings are run down into the chamber, making in it a total of 6 cc. of solution.

Liberation and measurement of CO_2 in the gas apparatus. The dissolved air and trace of CO_2 in the solution are extracted by evacuating the chamber and shaking for two minutes. The extracted gases are ejected as described on page 279. Any liquid reaching the cup is allowed to flow back into the chamber. The wall of the cup is washed down with 1 cc. of acidified 0.15 N $KMnO_4$, which is then allowed to run into the chamber. The chamber is evacuated and, with the mercury somewhat below the 50-cc. mark, is shaken for three minutes. In this time the oxalic acid is oxidized to CO_2 and the latter is extracted from solution. The mercury surface is now set at the 50-cc. mark and the shaking is continued for one minute. The precipitate of partly reduced manganese oxide which first forms may entirely disappear during the last minute, leaving a water clear solution, because of the reducing effect of the mercury in the chamber. The mercury surface during the first three minutes must be so low in the tube at the bottom of the chamber that no portions of mercury are whirled up into the bulb of the chamber during the shaking. If mercury does get into the bulb at this time the permanganate may be all reduced by the mercury before the oxalic acid is oxidized entirely to CO_2.

After the CO_2 is extracted the mercury is allowed to ascend in the chamber, with the precautions previously outlined on page 277 for "Adjustment of gas volume in CO_2 analyses." If the sample represents 1 cc. or less of serum (0.1 mg. or less of Ca) the gas volume is brought to 0.5 cc. for the p_1 reading. If the sample represents 2 cc. or more of serum (or over 0.2 mg. of Ca) it is preferable to read p_1 with the gas at 2-cc. volume.

After the p_1 reading is recorded the cock leading to the leveling bulb is opened and the bulb is placed at the medium level shown in figure 37, so that gas in the chamber is under slight negative pressure. One cubic centimeter of 5 N sodium hydroxide followed by a little mercury is then admitted to the chamber to absorb the CO_2. The p_2 reading is finally taken with the same gas volume in the chamber as at the p_1 reading.

Determination of c correction. A blank analysis is performed in which 2 cc. of 1 N sulfuric acid and 4 cc. of water are placed in the chamber of the manometric apparatus and analyzed as above described under "Liberation and measurement of CO_2." The $p_1 - p_2$ difference obtained is the c correction, which is partly due to traces of CO_2 dis-

TABLE 46

FACTORS BY WHICH MILLIMETERS P_{CO_2} ARE MULTIPLIED TO CALCULATE OXALIC ACID OR CALCIUM (FROM VAN SLYKE AND SENDROY (63))

In all cases it is assumed that the volume S of solution extracted in the Van Slyke-Neill chamber is 7.0 cc., the volume of the chamber is 50 cc., and the sample analyzed represents 1 cc. of serum or other fluid.

TEMPERATURE	FACTORS TO GIVE MILLIGRAMS OF Ca IN SAMPLE ANALYZED		FACTORS TO GIVE* MILLIGRAMS OF Ca PER 100 CC. IN SOLUTION ANALYZED, WHEN SAMPLE REPRESENTS 1 CC.		FACTORS TO GIVE MILLI-EQUIVALENTS OF Ca OR OXALIC ACID PER LITER, WHEN SAMPLE REPRESENTS 1 CC.		FACTORS TO GIVE MILLIMOLES OF Ca OR OXALIC ACID PER LITER, WHEN SAMPLE REPRESENTS 1 CC.	
	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.
°C.								
10	0.000715	0.002804	0.0716	0.2804	0.0357	0.1399	0.01785	0.0700
11	09	2780	09	0.2779	54	87	770	694
12	03	56	03	55	51	77	755	88
13	697	34	0.0697	35	48	65	740	83
14	92	13	91	13	45	54	725	77
15	86	2693	86	0.2691	43	43	713	72
16	81	71	81	71	40	33	700	67
17	76	51	76	50	37	23	0.01688	62
18	71	31	71	32	35	14	676	57
19	66	13	66	14	33	05	664	53
20	62	2595	61	0.2596	30	0.1296	652	48
21	57	78	57	78	28	87	640	44
22	53	61	53	60	26	78	629	39
23	48	43	49	43	24	70	618	35
24	44	26	44	26	21	61	607	31
25	40	10	39	09	19	52	0.01597	26
26	36	2493	35	0.2493	17	44	587	22
27	32	77	31	77	15	36	577	18
28	28	63	27	63	13	29	567	15
29	24	48	24	49	12	22	558	11
30	20	34	21	35	10	15	550	08
31	17	21	18	21	08	08	542	04
32	14	08	15	07	07	02	533	01
33	10	2394	11	0.2394	05	0.1195	524	0.0598
34	07	81	07	82	03	89	515	95

For samples other than 1 cc. the factors in the last 6 columns are divided by the volume of the sample in cubic centimeters.

* To calculate grams Ca per liter (= mg. per cc.), the factors in these 2 columns are divided by 100.

solved in the added permanganate and partly to the effects of the added 5 N sodium hydroxide solution on the mercury level in the chamber. The c value is ordinarily about 5 mm. when the gas is measured at 0.5 cc. volume, 1 or 2 mm. at 2 cc. volume.

Calculation. The pressure, P_{CO_2}, due to CO_2 from oxalic acid is calculated as

$$P_{CO_2} = p_1 - p_2 - c.$$

The P_{CO_2} value thus obtained is multiplied by the proper factor in table 46 to estimate calcium. Table 46 has been computed on the assumption that each molecule of oxalic acid yields 99.4 per cent of 2 molecules of CO_2 under the conditions of analysis, as was found by Van Slyke and Sendroy (63).

Cleaning chamber of manometric apparatus. After each analysis the apparatus is cleaned, in the manner described on page 236, except that ***lactic acid is not used.*** The chamber is washed first with water, then with 1 N sulfuric acid. It is important for this analysis that the chamber be clean and free of organic matter. ***Lactic acid is oxidized with formation of CO_2 by permanganate; hence it is essential that no traces of it be present.***

CALCIUM IN URINE

McKay and Butler's adaptation of Van Slyke and Sendroy method (unpublished)

The calcium is precipitated as oxalate under the conditions defined by McCrudden and utilized by Shohl and Pedley (see p. 763). The precipitate is washed and the oxalic acid in it determined as by the method of Van Slyke and Sendroy described above.

The preliminary oxidation of the urine with persulfate, which Shohl and Pedley found necessary when permanganate titration was used in determining the oxalate, is, except for albuminous urines, unnecessary when the gasometric method is used. Such amounts of uric acid as contaminate the precipitate were found by MacKay and Butler to yield no significant amounts of CO_2 in the gasometric determination.

In perfectly fresh albuminous urines the method can be used without preliminary oxidation of organic matter. But if albuminous urine has stood even for a day an unfilterable turbidity develops, and oxalate precipitates only part of the calcium. The rest is apparently held in colloidal solution.

Before the calcium is precipitated, such urines are oxidized with persulfate as described for Shohl and Pedley's method in chapter 25.

Reagents

Saturated ammonium oxalate, approximately 1 N sulfuric acid, approximately 0.15 N potassium permanganate, approximately 5 N sodium hydroxide, 20 per cent sodium acetate, as described above for blood calcium determination.

Methyl red, 0.02 per cent solution in 50 per cent alcohol.

Ammonium persulfate, solid, for preliminary oxidation of albuminous urines.

Procedure

An amount of non-albuminous urine, usually from 3 to 10 cc., containing not over 1 mg. of calcium is placed in a centrifuge tube and brought to 10-cc. volume. Two drops of methyl red solution are added, and concentrated hydrochloric acid a drop at a time until the urine turns deep red. Then 1 or 2 drops more are added. If the solution is not clear the tube is heated in a beaker of boiling water for five minutes to dissolve any precipitate of calcium phosphate that may be present. One cubic centimeter of saturated ammonium oxalate solution is then added. The mixture is then brought to a pH between 4.8 and 5.2 by adding sodium acetate solution a drop at a time until the color of the urine is neither red nor yellow, but orange. The tube is stoppered, shaken vigorously for several minutes, and let stand at least a half-hour. The supernatant liquid is drawn off and the precipitate washed as described above on page 771. The precipitate is then dissolved in sulfuric acid and the oxalic acid is determined as described above for blood calcium determination. In the present case the pressure readings are taken with the gas volume at 2 instead of 0.5 cc., unless the Ca content of the urine is unusually slight.

If the urine contains albumin, a portion of 50 cc. or less is oxidized with ammonium persulfate, as described on page 763. The oxidized urine is neutralized to methyl red with ammonia, then concentrated hydrochloric acid is added by drops until the color turns red again. The urine is then diluted up to its original volume, or to twice that volume, and a sample is transferred to a centrifuge tube. It is brought to 10-cc. volume, treated with ammonium oxalate and sodium acetate, and the analysis is carried on as above described.

Calculation

$$\text{Grams of Ca per liter urine} = \frac{(p_1 - p_2 - c) \times f_{mg.}}{\text{cc. urine in sample}}$$

$$\text{Milli-equivalents of Ca per liter urine} = \frac{(p_1 - p_2 - c) \times f_{m.Eq.}}{\text{cc. urine in sample}}$$

$f_{mg.}$ is the factor in table 46 "to give milligrams of Ca in sample analyzed." $f_{m.Eq.}$ is the factor in table 46 "to give milli-equivalents of Ca per liter when sample represents 1 cc."

LACTIC ACID IN BLOOD. AVERY AND HASTINGS (3A)

This method is based on the fact that permanganate in strongly acid solution oxidizes lactic acid at room temperature with the production of 1 molecule of CO_2 from 1 molecule of lactic acid. (See discussion in Chapter 16). Slight amounts of CO_2 are also formed from other organic substances that are present in the Folin-Wu blood filtrate and are not removed by treatment with copper sulfate and lime. The error from such sources leads to results approximately 0.5 mM. per liter (4.5 mg. per 100 cc.) higher than by the Friedemann-Cotonio-Shaffer distillation method described in chapter XVI. The difference is so constant that Avery and Hastings found that when 0.5 mM. was subtracted from their blood lactic acid values the results thus corrected agreed, within usually 0.1 mM. and always 0.2 mM., with results by the Friedemann-Cotonio-Shaffer method.

The advantages of the gasometric method over the distillation methods are the speed and simplicity of the gasometric determination and the lack of need for special equipment other than the manometric blood gas apparatus. The disadvantage of the gasometric method lies in the fact than an empirical correction must be subtracted for the amount of CO_2 yielded by substances other than lactic acid. The correction appears to be so constant, however, that its does not introduce a significant error.

Of substances which might be present in blood, Avery and Hastings find that the following yield no CO_2 when treated as in this method: glycine, alanine, beta-hydroxybutyric acid, and urea. Those yielding a small amount of CO_2, but not reacting quantitatively, are uric acid and glucose. Those which react quantitatively with permanganate are acetoacetic and pyruvic acids. Glucose is removed in the analysis. The amount of acetoacetic and uric acid in ordinary blood was found insufficient to measurably affect the results. Pyruvic acid can hardly be present in a concentration of 0.5 mM. The substances yielding this amount of CO_2 in excess of the lactic acid are therefore at present unknown.

Reagents

10 N sulfuric acid.

Permanganate solution: To 100 cc. of 0.1 N potassium permanganate solution are added 10 cc. of 1 N sulfuric acid. The CO_2 in the solution is removed by shaking in an evacuated flask. The solution is then resaturated with air.

Sodium tungstate, 10 per cent, and N/12 sulfuric acid, as used in Haden's modification of the Folin and Wu procedure for the precipitation of blood proteins (see p. 66).

A 14 per cent solution of crystalline copper sulfate ($CuSO_4 \cdot 5\ H_2O$).

A 14 per cent suspension of calcium hydroxide.

5 N sodium hydroxide.

Procedure

To 1 cc. of blood or serum in a 15 cc. centrifuge tube are added 1 cc. of 10 per cent sodium tungstate and 8 cc. of N/12 sulfuric acid. The mixture is stirred with a glass rod at intervals during fifteen minutes for whole blood, or five minutes for serum, and is centrifuged. Seven cubic centimeters of the supernatant liquid are removed to a second 15 cc. centrifuge tube and 1 cc. each of 14 per cent copper sulfate and 14 per cent calcium hydroxide suspension are added. This treatment precipitates glucose completely (see reference 19 of chapter XVII). After agitating at intervals for thirty minutes, the tube is centrifuged and 6 cc. of the supernatant solution are pipetted into a third tube. Here the solution is acidified with one drop (approximately 0.04 cc.) of 10 N sulfuric acid and allowed to stand for one hour in order that calcium tungstate may precipitate.

When more than 1 cc. of blood is available, 2 or 3 cc. are used with corresponding multiples of the quantities of reagents so that enough filtrate for duplicate analyses is obtained.

Five cubic centimeters of the final filtrate followed by 1 cc. of 10 N sulfuric acid are introduced into the extraction chamber of the manometric apparatus. The stop-cock is sealed and the dissolved gases are removed from solution by shaking one minute with the mercury at the 50-cc. mark. The gases are ejected as described on page 279. The extraction and ejection are repeated once to remove the last traces of CO_2.

One cubic centimeter of the potassium permanganate solution is now added and the solution is shaken slowly, with the mercury just below the 50-cc. mark, for four minutes. After the first two minutes the shaking is interrupted long enough to run the solution up to the top of

the tube and back, in order to wash down the sides. Until the oxidation of the lactic acid is completed (three or four minutes) the shaking must be done with the mercury level so low in the tube at the bottom of the chamber that no mercury globules whirl about the chamber bulb; otherwise the mercury may reduce the permanganate before all the lactic acid is oxidized.

TABLE 47

FACTORS FOR CALCULATION OF MILLIMOLS OF LACTIC ACID PER LITER OF BLOOD, OR MILLIGRAMS PER 100 CC., FROM PRESSURE OF CO_2 (FROM AVERY AND HASTINGS (3A))

TEMPERATURE	FACTOR f	$9f$
°C.		
15	0.0882	0.794
16	75	87
17	68	81
18	62	76
19	56	70
20	50	65
21	44	60
22	38	54
23	32	49
24	26	43
25	20	38
26	15	33
27	10	29
28	05	24
29	01	21
30	0.0797	17

Sample equivalent to 0.389 cc. blood. Volume of solution extracted in chamber = 7 cc. Volume at which CO_2 is measured = 0.5 cc. f gives millimoles lactic acid per liter of blood. $9f$ gives milligrams of lactic acid per 100 cc. of blood.

The pressure, p_1, is read with the gas in the chamber at the 0.5-cc. mark, and the solution lowered and shaken again for one minute. A second reading is then made and, if this fails to check, the one-minute shakings are repeated until a constant reading is obtained. This usually occurs after one or two trials.

The solution is then allowed to go to the top of the chamber and 3 cc. of 5 N sodium hydroxide are admitted to absorb the CO_2. The surface

of the solution is then lowered a short distance below the 2-cc. mark and allowed to stand one minute for drainage. The pressure of gases minus the CO_2, p_2, is now read with the gases at 0.5 cc. volume. The difference between p_1 and p_2 represents the pressure of CO_2 liberated from the sample and reagents.

A blank determination is carried out as described above except that 1 cc. of distilled water is substituted for the blood or serum. The $p_1 - p_2$ value found in the blank is designated as c. It includes the corrections for the CO_2 liberated from the reagents and for the change in manometer reading produced by the addition of the 3 cc. of alkali.

Special points in technique

If the blood is not to be mixed with the Folin-Wu tungstic acid solution within three minutes after it is drawn, the receiving vessel should be provided with enough NaF or NH_4F to make a 1 per cent solution in the blood. Otherwise formation of lactic acid by glycolysis in the shed blood may appreciably affect the results. After treatment of the blood with tungstic acid and copper hydroxide the filtrate may be kept in a refrigerator for period up to two days with no appreciable change.

Throughout the handling of the solutions care should be taken that no foreign oxidizable material is introduced. Cork stoppers should not be used. Even the best washed filter paper yields substance which produces CO_2 when treated with permanganate under the conditions of the analysis. Hence centrifugation is used in place of filtration to separate precipitates from supernatant solutions.

Calculation

$$\text{Millimoles of lactic acid per liter blood} = [(p_1 - p_2 - c) \times f] - 0.5$$

$$\text{Milligrams of lactic acid per 100 cc. blood} = [(p_1 - p_2 - c) \times 9f] - 4.5$$

The values of f, and $9f$ for temperatures from 15° to 30°C. are given in table 47.

POTASSIUM. KRAMER AND GITTLEMAN (19A)

Potassium is precipitated as cobalti-nitrite. The precipitate is decomposed with alkali.

$$K_2NaCo(NO_2)_6 + 3\ NaOH = 2\ KNO_2 + 4\ NaNO_2 + Co(OH)_3$$

The solution of nitrites thus obtained is washed into the chamber of the

manometric apparatus, and the nitrite is determined by reaction in acid solution with urea.

$$2\ HNO_2 + CO(NH_2)_2 = 2\ N_2 + CO_2 + 3\ H_2O.$$

Kramer and Gittleman state that the error does not exceed 0.01 mg. of K in the sample analyzed, or 1 mg. of K per 100 cc. serum.

The 0.2 mg. of potassium in 1 cc. of normal serum yields by these reactions enough N_2 to give about 140 mm. of pressure to read at 2-cc. volume.

Reagents

Sodium cobalti-nitrite reagent (see p. 748, Chapter XXIV).

1 N sodium hydroxide, approximate.

10 per cent urea solution.

8 N sulfuric acid, approximate. Twenty-five cubic centimeters of concentrated sulfuric acid of 1.83 to 1.84 specific gravity diluted to 100 cc.

10 N sodium hydroxide, approximate. Forty grams of NaOH dissolved and made up to 100 cc.

Procedure

One cubic centimeter of the serum, or solution containing about 0.2 mg. of K, is measured into a 15-cc. graduated centrifuge tube, containing 1 cc. of water. One cubic centimeter of the sodium cobalti-nitrite reagent is added slowly, drop by drop, and the solution thoroughly mixed after each drop. At the end of forty-five minutes the volume is made up to 5 cc. with water and the contents are mixed again and centrifuged for one-half hour at a speed of about 1800 revolutions per minute.

All but 0.3 cc. of the supernatant fluid is pipetted off by the technique described on page 771 in the calcium chapter for washing calcium oxalate. Five cubic centimeters of water are allowed to run in slowly. The stream is controlled in such a way that the added water is thoroughly mixed with the residual reagent, but the precipitate is not disturbed. The tube is centrifuged for five minutes. The procedure is repeated twice, making three washings in all. In the third washing, the water is added in such a way as to gently agitate the upper portion of the precipitate. The supernatant fluid from the last washing must be colorless. After this has been pipetted off, 0.7 cc. of approximately normal sodium hydroxide is added and the precipitate is thoroughly mixed with a fine glass rod. The tube is heated in a boiling water bath for about five minutes, during which a brownish flocculent precipitate

of cobalt hydroxide settles on the bottom of the tube. The solution is cooled and transferred to the chamber of the gas apparatus. The remainder of the precipitate is washed into the chamber with 2 cc. of 10 per cent urea solution. One cubic centimeter of approximately 8 N sulphuric acid is let in, after which a vigorous evolution of gas takes

TABLE 48

FOR CALCULATION OF SERUM POTASSIUM

Serum sample = 1 cc. P_{N_2} is read with gas at 2 cc. volume

TEMPERATURE	FACTOR BY WHICH P_{N_2} IS MULTIPLIED TO GIVE		
	Milli-equivalents of K per liter serum	Milligrams of K per 100 cc. serum	Milligrams of K in sample analyzed
°C.			
15	0.0371	0.1451	0.001451
16	69	45	45
17	68	40	40
18	67	35	35
19	66	30	30
20	64	25	25
21	63	20	20
22	62	14	14
23	60	09	09
24	58	04	04
25	58	00	00
26	57	0.1395	0.001395
27	56	90	90
28	54	85	85
29	53	80	80
30	52	75	75
31	51	70	70
32	49	65	65
33	48	60	60
34	47	56	56

place. The mercury is lowered almost to the bottom of the 50-cc. bulb, the apparatus is sealed, and the level of the mercury then lowered to the 50-cc. mark. After shaking for about five minutes, the mercury is raised again so that a space of several cubic centimeters is left between the water meniscus and the 2-cc. mark. Three cubic centimeters of approximately 10 N sodium hydroxide are permitted to flow gradually

into the chamber to absorb the CO_2 present. The cock is sealed again, the gas volume is reduced to the 2-cc. mark, and the pressure p_1 is read.

A blank analysis is done, in which 1 cc. of the 1 N NaOH is put through the above procedure. The pressure reading of the blank is p_0.

The pressure due to nitrogen from the nitrite is

$$P_{N_2} = p_1 - p_0$$

Calculation

To obtain the potassium content of the serum P_{N_2} is multiplied by a factor from table 48.

By the reactions given above each equivalent of K precipitates 3 equivalents of NO_2, which yield 3 moles of N_2 gas. To calculate millimoles of K per liter of serum, when the sample is 1 cc., one therefore multiplies the observed P_{N_2} by one-third the factor in table 30 for calculating millimoles N_2 per liter from analysis of 1-cc. samples. These factors are multiplied by $39.1 \times \frac{100}{1000}$ to obtain its factor for calculation of serum potassium in milligrams per 100 cc. The analyst should read the discussion of the cobalti-nitrite method in chapter 24, page 746.

MICRO DETERMINATION OF CARBON BY WET COMBUSTION AND ITS APPLICATION TO DETERMINATION OF LIPOIDS. (BACKLIN (3B))

Carbon in organic matter is burned to CO_2 by heating with sulfuric acid and silver dichromate, silver acting as catalyzer to accelerate the oxidation. The CO_2 is transferred to the chamber of the gas apparatus and there absorbed with sodium hydroxide solution. The absorbed CO_2 is then determined as in blood analyses. Backlin states that the yield of CO_2 is quantitative, and that the results are more accurate than can be obtained by the empirical chromate reduction method of Bang, even in the present improved modification of Bloor (p. 496).

Backlin has described the method for use with the volumetric type of apparatus. Because more accurate results are obtainable with the manometric apparatus, however, we shall adapt the description to the latter. The determination requires about twenty-five minutes.

In applying the method to lipoid determination, the lipoid material is first isolated by one of the methods described in chapter 9, with the fats in the form of either neutral fats or fatty acids. The material is redissolved and aliquot parts of not over 1 mg. are used for combustion.

Apparatus

This consists of the combustion tube *A* (fig. 61) and its connections. The tube is about 20 by 190 mm. in size, and of Pyrex or other hard glass.

The stopper which closes it at the top may be of rubber, but it is better to have a ground-in glass stopper. After repeated use the lower side of a rubber stopper is attacked by the fumes, and small particles of rubber may then fall into the combustion mixture and cause high results. Through the stopper pass the connecting tube to the gas chamber and the stem of the dropping funnel *M*, which is marked at 1-cc. intervals to contain 5 cc. The

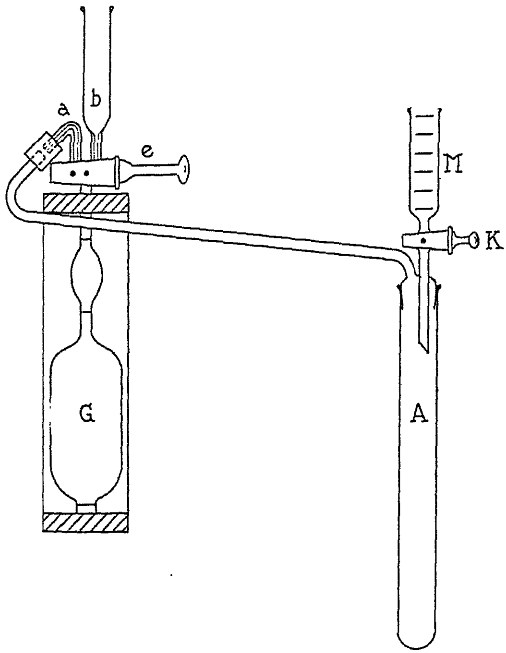


Fig. 61. Apparatus arranged for determination of carbon by wet combustion. Modified from Backlin (3a) to fit manometric apparatus.

connecting tube, of 4 mm. bore is joined glass on glass by pure gum tubing to the capillary inlet *a* of the manometric chamber. Ground glass joint and stopper K are lubricated with concentrated sulfuric acid.

Reagents

Silver dichromate and sulfuric acid. Five grams of silver nitrate are dissolved in 25 cc. of water. Five grams of potassium dichromate are dissolved in 50 cc. of water. The two solutions are mixed. The precipitate of silver dichromate is centrifuged, washed twice with distilled water, and finally dissolved in 500 cc. of concentrated sulfuric acid. Before it is used this solu-

tion is heated two hours on the steam bath and let stand three days, in order to insure destruction of any organic matter present.

Approximately 0.5 N NaOH. Water is freed of CO_2 by adding one drop of 0.1 N hydrochloric acid per 100 cc. and boiling. To 100 cc. of this water are added 3 cc. of the CO_2-free 18 N NaOH described under "General reagents" on page 233. The solution is at once drawn into a burette protected at the top by a soda-lime tube, and provided with a long tip at the bottom, as shown in figure 52.

Approximately 2 N lactic acid. Twenty cubic centimeters of concentrated lactic acid diluted to 100 cc.

Approximately 5 N NaOH. Described under "General reagents."

Procedure

Removal of solvent. Dissolved in petroleum ether or alcohol, the sample of lipoid, which should not exceed 1 mg., is placed in test tube *A* (fig. 61). The solvent is driven off by heating the open tube in a water bath. The last traces of petroleum ether are removed by leading through a current of air for a few minutes. To remove the last traces of alcohol, after the material is apparently dry a few drops of water are added, and an air current is passed through the heated tube. Cholesterol digitonide may be precipitated, washed, and dried in tube *A*, as described in the last section of the lipoid chapter. For the precipitation and washing tube *A* should, in this case, be a conical centrifuge tube.

Combustion. The tube is connected with the gas chamber as shown in figure 61. Cock *K* is closed (it is best lubricated with a little concentrated sulfuric acid), and cock *e* is turned to connect *G* and *A*. By lowering the leveling bulb air is drawn from *A* over into *G*, and by properly manipulating cock *e*, is ejected from the system through the other outlet of cock *e*. This is repeated, if necessary, until the pressure in the system is such that, with *G* fall of mercury, the mercury in the manometer is about 500 mm. higher than that in the chamber. One or 2 cc. of mercury are run up into cup *b* of the apparatus, and 0.5 N NaOH is run in from a burette, as shown in figure 52, until the solution reaches exactly to the 2-cc. mark in the chamber. Cock *e* is sealed and the mercury is drawn to the lower third of *G*. The mercury is maintained at this level, not by the usual means of closing the cock to the leveling bulb, but by hanging the leveling bulb at a proper height and leaving the connecting cock open. Then, if small explosions occur in the combustion, displacement of mercury from *G* can take up some of their force.

Cock *e* is then turned to connect *G* and *A*, and 5 cc. of the silver

chromate-sulfuric acid mixture are run into A, with caution to avoid letting in air after the fluid. With a micro burner the mixture in A is heated cautiously to boil (*protect eyes by goggles*) until white fumes appear. Evolution of CO_2 gas then begins. During the heating the ring stand on which A is clamped is shaken at times in order to throw the acid mixture about the walls at the bottom of A and wash back drops of material that may have spattered onto the walls. The mixture is heated for 10 minutes just enough to keep it boiling, but not enough to fill the tube with white fumes. The flame is removed and the mixture is permitted to cool for a minute or two. The manometric leveling bulb is then repeatedly lowered and raised, so that the mercury rises and falls in the broad part of G. Thereby the gas from A is drawn over into G, where the CO_2 is absorbed by the alkali.

Determination of CO_2. After the combustion and absorption of CO_2 are complete cock e is closed and the combustion tube is disconnected from the gas chamber. A little mercury from the hand is drawn into a to seal that outlet of the cock. All the gases in G are then ejected, as described on page 279.

Two cubic centimeters of 1 N lactic acid are placed in the cup of the apparatus, and 1.5 cc. are run into the chamber. The mercury is lowered to the 50-cc. mark, the chamber is shaken two minutes, and the gas volume is brought to 2 cc. with the precautions for CO_2 determinations described on page 277. Reading p_1 on the manometer is taken. 0.5 cc. of the 5 N NaOH is then slowly admitted to absorb the CO_2, the fluid in the chamber being allowed to rise as close to the cock as it will. The meniscus is then brought again to the 2-cc. mark and reading p_2 is taken.

A blank analysis is performed with the reagents alone. The $p_1 - p_2$ reading obtained from the blank is the c correction.

Calculation

$$\text{Milligrams of } CO_2 \text{ in sample} = (p_1 - p_2 - c) \times f_{CO_2}$$

$$\text{Milligrams of carbon in sample} = (p_1 - p_2 - c) \times f_C$$

$$\text{Milligrams of lipoid in sample} = (p_1 - p_2 - c) \times f_{lipoid}$$

The different factors, $f\,CO_2$ etc., are given in table 49. Those for CO_2 are taken from table 9 of Van Slyke and Sendroy (62). Those for carbon are obtained from the CO_2 factors by multiplying by $12/44 = 0.2727$. The factor for each lipoid is obtained by multiplying the carbon factor by $100 \div$ (per cent C in lipoid). The carbon contents are: Cholesterol, 83.9 per cent; palmitic, stearic and oleic acids, 75.0, 76.0, and 76.6 per cent;

tripalmitin, tristerin, triolein, 75.9, 76.8, and 77.4 per cent. The mean carbon content for the above three free fatty acids that chiefly comprise animal fats is 75.9 per cent and of the fats 76.7, the maximum deviations being within ±1 per cent of these values. Hence the percentages 75.9 and 76.7 are used in calculating the "fatty acid" and "neutral fat" factors of table 49.

TABLE 49

For Calculation of Carbon Dioxide, Carbon, and Lipoids from P_{CO_2} Obtained After Wet Combustion of Organic Substances

$s = 3.5$ cc. $a = 2$ cc.

Temperature	Factors by which P_{CO_2} is multiplied to give						
	Milligrams of CO_2	Milligrams of C	Milligrams of cholesterol	Milligrams of cholesterol when the digitonid is oxidized	Milligrams of fatty acids	Milligrams of neutral fat	Milligrams of mixed plasma lipoids
°C.							
15	0.00540	0.001474	0.001756	0.000579	0.001941	0.001921	0.00187
16	37	66	47	76	31	11	6
17	34	58	37	72	21	01	5
18	32	50	28	69	11	0.001890	4
19	29	42	18	66	00	80	3
20	26	35	10	63	0.001891	71	2
21	23	27	01	60	81	60	1
22	21	20	0.001692	57	71	51	0
23	18	12	83	54	61	41	0.00179
24	15	05	74	52	52	32	8
25	13	0.001398	66	49	42	23	8
26	10	91	58	46	33	14	7
27	08	85	50	44	25	06	6
28	05	78	42	41	16	0.001797	5
29	03	72	35	39	08	89	4
30	01	65	27	36	0.001799	80	
31	0.00499	59	20	34	91	72	3
32	96	53	13	31	84	65	2
33	94	48	06	29	76	57	1
34	0.00492	0.001342	0.001599	27	0.001769	50	0.00170

The factor for mixed plasma lipoids is an approximate one estimated by assuming, as does Bloor (reference 10, chapter 9), that the mixture consists of 2 parts of fat to 1 of cholesterol. The mixed lipoid factors can be used when the cholesterol is mixed with either the neutral fats or with the fatty acids obtained after saponification, as in Stoddard's method, since the factors for neutral fats and fatty acids differ by only 1 per cent. For calculation of cholesterol, $C_{27}H_{46}O$, from the carbon determined in digitonin cholesteride,

$C_{27}H_{46}O \cdot C_{55}H_{94}O_{28}$, which has 61.9 per cent of carbon and 24.3 per cent of cholesterol (Windaus, 66) the carbon factor is multiplied by $\frac{100}{61.9} \times 0.243 = 0.3926$.

Dr. Esben Kirk in the laboratory of one of the writers has made preliminary tests of Backlin's method, with addition of the following points to the technique as adapted to the manometric apparatus. The heating must not be intense enough to drive fluid from condensed fumes into G. It is desirable to have small bulbs blown into the tube connecting A and G to condense any such fluid. The dropping funnel M and cock K are not necessary, at least for combustion of ordinary non-volatile substances. The sulfuric-dichromate reagent is added cold to the dried sample in A, which is at once connected as in figure 61. The manipulation is simplified by making tube A of smaller dimensions, viz. 18 by 125 mm. outside measurement and about 25 cc. capacity. With this the amount of reagent used is reduced to 2 cc. and the sample to not over 0.3 mg. of carbon. In determination of the CO_2 absorbed by the alkali in G, it is preferable to use lactic rather than sulfuric acid to set the CO_2 free. Sulfuric frees SO_2 that may accompany the CO_2, while lactic acid is too weak to decompose sulfite.

BIBLIOGRAPHY

1. Abeloos, M.; Barcroft, J.; Cordero, N.; Harrison, T. R., and Sendroy, J., Jr.: The measurement of the oxygen capacity of haemoglobin. *J. Physiol.*, 1928, **66**, 262.
2. Austin, J. H.: A note on the estimation of carbon dioxide in serum in the presence of ether by the Van Slyke method. *J. Biol. Chem.*, 1924, **61**, 345.
3. Austin, J. H.; Cullen, G. E.; Hastings, A. B.; McLean, F. C.; Peters, J. P., and Van Slyke, D. D.: Studies of gas and electrolyte equilibria in blood. I. Technique for collection and analysis of blood, and for its saturation with gas mixtures of known composition. *J. Biol. Chem.*, 1922, **54**, 121.

3a. Avery, B. F., and Hastings, A. B.: A gasometric method for the determination of lactic acid in blood. *J. Biol. Chem.*, 1931, **94**, 273.

3b. Backlin, E.: Eine Mikromethode zur quantitativen Bestimmung der Lipoide. *Biochem. Z.*, 1930, **217**, 483.

3c. Baernstein, H. D.: The gasometric determination of cysteine and cystine. *J. Biol. Chem.*, 1930, **89**, 125.

4. Barcroft, J., and Haldane, J. S.: A method of estimating the oxygen and carbonic acid in small quantities of blood. *J. Physiol.*, 1902, **28**, 232.
5. Barcroft, J., and Higgins, H. L.: The determination of the constants of the differential blood-gas apparatus. *J. Physiol.*, 1911, **42**, 512.
6. Barcroft, J., and Roberts, F.: Improvements in the technique of blood-gas analysis. *J. Physiol.*, 1909–1910, **39**, 429.
7. Bock, J. C.: The amino-acid nitrogen of the blood of various species. *J. Biol. Chem.*, 1917, **29**, 191.
8. Conant, J. B.: An electrochemical study of hemoglobin. *J. Biol. Chem.*, 1923, **57**, 401.

8a. Eisenman, Anna J.: A gasometric method for the determination of pH in blood. *J. Biol. Chem.*, 1926, **71**, 611.

9. Fieser, L. F.: A new absorbent for oxygen in gas analysis. *J. Am. Chem. Soc.*, 1924, **46**, 2639.

10. Fiske, C. H.: A method for the estimation of total base in urine. *J. Biol. Chem.*, 1922, **51**, 55.

11. Folin, O., and Svedberg, A.: The sugar in urine and in blood. *J. Biol. Chem.*, 1926, **70**, 405.

11a. Gamble, J. L.: Carbonic acid and bicarbonate in urine. *J. Biol. Chem.*, 1922, **51**, 295.

12. Gréhant, M. and Quinquaud, E.: Mesure du volume de sang contenu dans l'organisme d'un mammifère vivant. *Compt. rend. Acad.*, 1882, **94**, 1450.

13. Guye, P. A., and Pintza, A.: Determination des densités des gaz anhydride carbonique, ammoniac, et protoxyde d'azote. *Mém. Soc. Phys. et Hist. nat. Genève*, 1908, **35**, 551.

14. Hagedorn, H. C., and Jensen, B. N.: Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. *Biochem. Z.*, 1923, **135**, 46.

15. Haldane, J. S.: A new apparatus for accurate blood-gas analysis. *J. Path. Bact.*, 1921, **23**, 443.

16. Harington, C. R., and Van Slyke, D. D.: On the determination of gases in blood and other solutions by vacuum extraction and manometric measurement. II. *J. Biol. Chem.*, 1924, **61**, 575.

16a. Hastings, A. B., and Avery: Manometric determination of lactic acid in blood. *J. Biol. Chem.*, 1931, **94**, 273.

16b. Hastings, A. B.; Neill, J. M.; Morgan, H. J., and Binger, C. A. L.: Blood reaction and blood gases in pneumonia. *J. Clin. Inv.*, 1924, **1**, 25.

17. Hastings, A. B.; Sendroy, J., Jr., and Van Slyke, D. D.: Studies of gas and eletrolyte equilibria in blood. XII. The value of pK′ in the Henderson-Hasselbalch equation for blood serum. *J. Biol. Chem.*, 1928, **79**, 183.

17a. Henderson, L. J., and Palmer, W. W.: On the intensity of urinary acidity in normal and pathological conditions. *J. Biol. Chem.*, 1913, **13**, 393.

18. Henderson, Y., and Smith, A. H.: Applications of gas analysis. V. Blood gas analysis. *J. Biol. Chem.*, 1918, **33**, 39.

19. Hiller, A.; Linder, G. C., and Van Slyke, D. D.: The reducing substances of the blood. *J. Biol. Chem.*, 1925, **64**, 625.

19a. Kramer, B., and Gittleman, I.: The gasometric determination of potassium. *Proc. Soc. Exp. Biol. and Med.*, 1926, **24**, 241.

20. Levene, P. A., and Van Slyke, D. D.: Gasometric determination of free and conjugated amino-acids in the urine. *J. Biol. Chem.*, 1912, **12**, 301.

21. Lundsgaard, C., and Möller, E.: On the determination of the total oxygen combining power of blood in the Van Slyke apparatus. *J. Biol. Chem.*, 1922, **52**, 377.

21a. Mainzer, F.: Zur manometrischen Messung der CO_2 nach Van Slyke. *Biochem. Z.*, 1930, **229**, 311.

21b. Mainzer, F., and Bruhn, M.: Über Löslichkeit, Dissoziation, und Spannung der Kohlensäure im Harn. *Biochem. Z.*, 1931, **230**, 395.

21c. Marshall, E. K., Jr.: The effect of loss of carbon dioxide on the hydrogen ion concentration of the urine. *J. Biol. Chem.*, 1922, **51**, 3.

22. Mirkin, A.: A gasometric method for the determination of urea nitrogen in the blood. *J. Lab. Clin. Med.*, 1922, **8**, 50.

23. Nicloux, M.: Dosage de traces d'oxyde de carbone dans l'air par la méthode au sang et considérations générales sur l'absorption de l'oxyde de carbone par l'hémoglobine en l'absence d'oxygenè. *Bull. soc. chim. de France*, 1925, **37**, 760.

24. NICLOUX, M., AND FONTÈS, G.: Sur quelques modes de formation de la méthémoglobine et sur son dosage. *Bull. soc. chim. biol.*, 1924, **6**, 728.

25. O'BRIEN, H. R., AND PARKER, W. L.: Solubility of carbon monoxide in serum and plasma. *J. Biol. Chem.*, 1922, **50**, 289.

26. OSBORNE, T. B.; LEAVENWORTH, C. S., AND BRAUTLECHT, C. A.: The different forms of nitrogen in proteins. *Am. J. Physiol.*, 1908, **23**, 179.

27. PARTOS, S.: Bestimmung des durch Urease zersetzten Harnstoffs aus der CO_2-Komponente des Zersetzungsproduktes. *Biochem. Z.*, 1921, **103**, 292.

28. PILAAR, W. M. M.: Determination of carbon monoxide in blood. *J. Biol. Chem.*, 1929, **83**, 43.

28a. PETERS, J. P.: Studies of the carbon dioxide absorption curve of human blood. III. A further discussion of the form of the absorption curve plotted logarithmically, with a convenient type of interpolation chart. *J. Biol. Chem.*, 1923, **56**, 745.

29. RAY, P. R., AND SEN, H. K.: Die Einwirkung von Hydrazin und Hydroxylamin auf Ferricyanide und neue Methoden zur Bestimmung von Hydrazin und Ferricyaniden. *Z. anorg. Chem.*, 1912, **76**, 380.

30. RIEGLER, E.: Eine gasvolumetrische Bestimmungsmethode der Sulfate, der Schwefelsäure, der Carbonate und der Kohlensäure. *Z. anal. Chem.*, 1902, **41**, 17.

31. SAYERS, R. R., AND YANT, W. P.: The pyrotannic acid method for the quantitative determination of carbon monoxide in blood and in air—its use in the diagnosis and investigation of cases of carbon monoxide poisoning. Bureau of Mines. Technical paper 373, Washington, D. C. 1925.

31a. SENDROY, J., JR.: Manometric determination of hemoglobin by the oxygen capacity method. *J. Biol. Chem.*, 1931, **91**, 307.

32. SENDROY, J., JR.; AND LIU, S. H.: Gasometric determination of oxygen and carbon monoxide in blood. *J. Biol. Chem.*, 1930, **89**, 133.

33. SENDROY, J., JR.; LIU, S. H., AND VAN SLYKE, D. D.: The gasometric estimation of the relative affinity constant for carbon monoxide and oxygen in whole blood at 38°. *Proc. XIIIth International Physiol. Congress*, 1929, page 242. *Am. J. Physiol.*, 1929, **90**, 511.

34. SHOHL, A. T.: A trap for the Van Slyke gas analysis apparatus. *J. Biol. Chem.*, 1923, **56**, 125.

35. SHOHL, A. T.: The effect of hydrogen ion concentration upon the determination of calcium. *J. Biol. Chem.*, 1922, **50**, 527.

36. SOMOGYI, M.: Notes on sugar determination. *J. Biol. Chem.*, 1926, **70**, 599.

37. SOMOGYI, M.: The distribution of sugar in normal human blood. *J. Biol. Chem.*, 1928, **78**, 117.

38. STADIE, W. C.: A mechanical shaker and other devices for use with the Van Slyke blood gas apparatus. *J. Biol. Chem.*, 1921, **49**, 43.

38a. STADIE, W. C., AND VAN SLYKE, D. D.: Studies of acidosis. XV. Carbon dioxide content and capacity in arterial and venous blood plasma. *J. Biol. Chem.*, 1920, **41**, 191.

39. STEHLE, R. L.: Gasometric determination of nitrogen and its application to the estimation of the non-protein nitrogen of blood. *J. Biol. Chem.*, 1920–21, **45**, 223.

40. STEHLE, R. L.: Note on the gasometric determination of nitrogen. *J. Biol. Chem.*, 1921, **47**, 11.

41. STEHLE, R. L.: The gasometric determination of urea in urine. *J. Biol. Chem.*, 1921, **47**, 13.

42. Tolstoi, E.: Glycolysis in bloods of normal subjects and of diabetic patients. *J. Biol. Chem.*, 1924, **60**, 69.

43. Van Slyke, D. D.: A method for quantitative determination of aliphatic amino groups. Applications to the study of proteolysis and proteolytic products. *J. Biol. Chem.*, 1911, **9**, 185. The quantitative determination of aliphatic amino groups. II. *J. Biol. Chem.*, 1912, **12**, 275.

The gasometric determination of aliphatic amino nitrogen in minute quantities. *J. Biol. Chem.*, 1913–14, **16**, 121.

43a. Van Slyke, D. D.: Improved methods in the gasometric determination of free and conjugated amino acid nitrogen in the urine. *J. Biol. Chem.*, 1913, **16**, 125.

44. Van Slyke, D. D.: Studies of acidosis. II. A method for the determination of carbon dioxide and carbonates in solution. *J. Biol. Chem.*, 1917, **30**, 347.

45. Van Slyke, D. D.: Gasometric determination of the oxygen and hemoglobin of blood. *J. Biol. Chem.*, 1918, **33**, 127.

46. Van Slyke, D. D.: The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. III. Gasometric determination of methemoglobin. *J. Biol. Chem.*, 1925, **66**, 409.

47. Van Slyke, D. D.: Gasometric micro-Kjeldahl determination of nitrogen. *J. Biol. Chem.*, 1927, **71**, 235.

48. Van Slyke, D. D.: Note on a portable form of the manometric gas apparatus, and on certain points in the technique of its use. *J. Biol. Chem.*, 1927, **73**, 121.

49. Van Slyke, D. D.: Determination of urea by gasometric measurement of the carbon dioxide formed by the action of urease. *J. Biol. Chem.*, 1927, **73**, 695.

50. Van Slyke, D. D.: Manometric determination of primary amino nitrogen and its application to blood analysis. *J. Biol. Chem.*, 1929, **83**, 425.

51. Van Slyke, D. D.: The manometric determination of urea in blood and urine by the hypobromite reaction. *J. Biol. Chem.*, 1929, **83**, 449.

52. Van Slyke, D. D., and Cullen, G. E.: A permanent preparation of urease, and its use in the determination of urea. *J. Biol. Chem.*, 1914, **19**, 211.

53. Van Slyke, D. D., and Cullen, G. E.: Studies of acidosis. I. The bicarbonate concentration of the blood plasma; its significance, and its determination as a measure of acidosis. *J. Biol. Chem.*, 1917, **30**, 289.

54. Van Slyke, D. D., and Hawkins, J. A.: A gasometric method for the determination of reducing sugars, and its application to the analysis of blood and urine. *J. Biol. Chem.*, 1928, **79**, 739.

55. Van Slyke, D. D., and Hawkins, J. A.: Gasometric determination of fermentable sugar in blood and urine. *J. Biol. Chem.*, 1929, **83**, 51.

56. Van Slyke, D. D., and Hiller, A.: Gasometric determination of hemoglobin by the carbon monoxide capacity method. *J. Biol. Chem.*, 1928, **78**, 807.

56*a*. Van Slyke, D.D., and Hiller, A.: Gasometric control of standard solutions for the Palmer hemoglobin method. *J. Biol. Chem.*, 1929, **84**, 211.

57. Van Slyke, D., and Hiller, A.: Gasometric determination of methemoglobin. *J. Biol. Chem.*, 1929, **84**, 205.

58. Van Slyke, D. D.; Hiller, A., and Berthelsen, K. C.: A gasometric micro method for determination of iodates and sulfates, and its application to the estimation of total base in blood serum. *J. Biol. Chem.*, 1927, **74**, 659.

59. Van Slyke, D. D., and Neill, J. M.: The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. *J. Biol. Chem.*, 1924, **61**, 523.

60. VAN SLYKE, D. D., AND ROBSCHEIT-ROBBINS, F. S.: The gasometric determination of small amounts of carbon monoxide in blood, and its application to blood volume studies. *J. Biol. Chem.*, 1927, **72**, 39.

61. VAN SLYKE, D. D., AND SALVESEN, H. A.: The determination of carbon monoxide in blood. *J. Biol. Chem.*, 1919, **40**, 103.

62. VAN SLYKE, D. D., AND SENDROY, J., JR.: Carbon dioxide factors for the manometric blood gas apparatus. *J. Biol. Chem.*, 1927, **73**, 127.

63. VAN SLYKE, D. D., AND SENDROY, J., JR.: Gasometric determination of oxalic acid and calcium, and its application to serum analyses. *J. Biol. Chem.*, 1929, **84**, 217.

63a VAN SLYKE, D. D., AND SENDROY, J., JR.: Studies of gas and electrolyte equilibria in blood. XV. Line charts for graphic calculations by the Henderson-Hasselbalch equation, and for calculating plasma carbon dioxide content from whole blood content. *J. Biol. Chem.*, 1928, **79**, 781.

63b VAN SLYKE, D. D., SENDROY, J., JR., HASTINGS, A. B., AND NEILL, J. M.: Studies of gas and electrolyte equilibria in blood. X. The solubility of carbon dioxide at 38° in water, salt solutions, serum, and blood cells. *J. Biol. Chem.*, 1928, **78**, 765.

63c VAN SLYKE, D. D., SENDROY, J., JR., AND LIU, S. H.: Manometric determination of CO_2 tension and pH in blood. *J. Biol. Chem.*, 1932, **95**, in press.

64. VAN SLYKE, D. D., AND STADIE, W. C.: The determination of the gases of the blood. *J. Biol. Chem.*, 1921, **49**, 1.

65. VAN SLYKE, D.D., WU, H., AND MCLEAN, F.C.: Studies of gas and electrolyte equilibria in blood. V. Factors controlling the electrolyte and water distribution in blood. *J. Biol. Chem.*, 1923, **56**, 765.

66. WINDAUS, A.: Ueber die Entgiftung der Saponine durch Cholesterin. *Ber. deutsch. chem. Ges.*, 1909, **42**, 238.

Über die quantitative Bestimmung des Cholesterins und der Cholesterinester in einigen normalen und pathologischen Nieren. *Zeit. f. physiol. chem.*, 1910, **65**, 110.

67. WONG, S. Y.: The use of persulfate in the estimation of nitrogen by the Arnold-Gunning modification of Kjeldahl's method. *J. Biol. Chem.*, 1923, **55**, 427.

CHAPTER VIII

SUGAR

GENERAL METHODS FOR GLUCOSE DETERMINATION

The sugar of which concentration changes in blood and urine require clinical study is, with exception of rare cases, glucose (see carbohydrate chapter in volume I).

The methods chiefly employed for the quantitative determination of glucose have depended upon three of its properties, its ability to reduce in alkaline solution salts of certain of the heavy metals or of nitroaromatic acids, its dextrorotation, and its generation of carbon dioxide when fermented with yeast. None of these properties is peculiar to glucose. Even CO_2 formation by yeast occurs from action of the latter on non-sugar substances (32, 33, 43). No method has been yet devised which has been proved to measure solely glucose in either blood or urine. The procedure which comes nearest to this goal is a combination of reduction and fermentation methods, measurement of the decrease in reduction which occurs when yeast acts under specified conditions.

For most clinical purposes, however, the reduction methods suffice. The content and variability of non-glucose reducing substances in blood and urine is low in comparison with the glucose increases that occur in glycosuria or glycemia. Consequently one can set certain limits for the maximum normal contents of total reducing substances and interpret the occurrence of greater contents as evidence of abnormal glucose concentrations. In special cases this interpretation may be doubtful, and a combined fermentation and reduction analysis is needed.

Polariscopic methods have never acquired general use in clinical laboratories. They are not adapted to determination of the small amounts of sugar found in blood, and they require the use of an expensive apparatus which would have as a rule little other use in such laboratories. It is the reduction methods that have been developed in infinite variety for blood and urine analyses.

These methods may be classified according to (1) the oxidizing agent, and (2) the analytical procedure for measuring the amount of such agent that is reduced.

The first oxidizing agent that acquired general use was the alkaline copper

sulfate solution introduced by Fehling in 1849 (14). The solution was made alkaline with sodium hydroxide, and tartrate was added to prevent the copper from being precipitated as cupric hydroxide. To this solution at boiling temperature Fehling added the sugar solution until all the copper was reduced and precipitated as red cuprous oxide, and the blue color of dissolved cupric salts disappeared from the solution. The alkaline copper tartrate solution did not keep well. In consequence it became the practice with later analysts to prepare the copper sulfate and the alkali tartrate as separate solutions which were mixed immediately before use. The determination of the end point was difficult, and innumerable modifications of the method have been published in which special technique or indicators are used to facilitate sharp detection of the end-point, or in which fixation of the end-point is avoided by using excess copper solution and determining by various methods of copper analysis either the Cu_2O precipitated or the excess of unreduced copper left in solution. Of such procedures the widest application appears to have been won by that of Bertrand (11), in which the cuprous oxide is filtered, and redissolved in an excess of acid ferric sulfate solution. An amount of Fe^{+++} equivalent to the Cu^+ is reduced by the reaction, $Cu^+ + Fe^{+++} = Cu^{++} + Fe^{++}$. The ferrous iron is titrated with permanganate. The method is accurate and convenient, and is still much used. For blood analyses, however, the amounts of cuprous oxide formed are too small to handle conveniently, and, also because of the small amounts involved, special precautions are required to avoid reoxidation of cuprous copper by air. Consequently in blood sugar methods means have been found to determine the reduced copper without isolation of the Cu_2O, and special precautions have been developed to avoid reoxidation by air. Also more stable reagent solutions have been devised, notably that of Benedict (5), and substances have been added to the reagent to make it less sensitive to the non-glucose reducing materials in blood and urine. These modifications will be discussed in connection with specific blood and urine methods below.

Picric acid as a sugar oxidizing agent was introduced into quantitative blood analysis in 1913 by Lewis and Benedict (6, 30) and was applied to urine by Hiller (27) and by Benedict and Osterberg (10). The reduction product, has a much more intense red color than picric acid, and is well suited for colorimetry. Sumner (41) used dinitrosalicyclic acid for urine sugar. At present these reagents have receded into the background before copper and ferricyanide, which appear, particularly when applied to blood filtrates, to be less liable to reduction by non-glucose substances and more readily adapted to micro-technique. Benedict (8, 9, 10) himself has abandoned picrate for copper reagents.

Ferricyanide for quantitative sugar determination was introduced by Hagedorn and Jensen (22) in 1923. It has over copper the advantages that the reduction product formed, ferrocyanide, remains in solution and can be easily determined in minute amounts by titrimetric, colorimetric, or gasometric methods, and furthermore that the ferrocyanide is not readily reoxidized by air, so that avoidance of error from this source requires no precautions. In consequence of these advantages ferricyanide methods have been rapidly replacing copper procedures, especially for blood analyses. Recently Folin (15, 16) has abandoned his own copper colorimetric method to devise a ferricyanide one.

Salts of other metals with variable valences, such as mercury and bismuth, can be used to oxidize glucose, and have been employed to some extent in its qualitative detection, but have not gained use for quantitative analysis.

The ability to reduce cupric salts and ferricyanide depends upon the presence of the aldehyde or ketone group in the sugar molecule. It is shared with glucose by other sugars which possess such groups. Thus for ferricyanide Hawkins (22) found that the following sugars showed the indicated reducing powers, that of an equal weight of glucose being taken as 100; mannose, 101; galactose, 79; fructose, 98; arbinose, 94; xylose, 100; maltose, 72; and lactose, 72. For copper reagents Bertrand (11) found that mannose, arabinose, and xylose show also approximately 100 per cent of the reducing power of glucose, galactose, 94; maltose, 55; and lactose, 70. Sucrose has no reducing power towards these reagents, because it has no free aldehyde or ketone groups. When hydrolyzed to invert sugar it has approximately the same reducing power as glucose.

Although the presence of an aldehyde or ketone group is necessary to render a sugar oxidizable by cupric or ferricyanide salts, the oxidation is not limited to the carbonyl group. It spreads further into the sugar molecule, to an extent varying with time, alkalinity, and other conditions. With Fehling's solution 1 mole of glucose reduces about 10 of cupric salt (13), of which 24 moles would yield the amount of oxygen required to oxidize the sugar completely to CO_2 and H_2O. Of ferricyanide 6 or 7 molecules under the conditions of analysis are reduced by 1 molecule of glucose (42).

Lactose is differentiated from glucose in that, while it is a reducing sugar, it is not fermented by yeast. This fact finds clinical application in ascertaining whether positive reduction tests encountered in the urine during and after pregnancy are due to lactose or glucose.

The procedures, gravimetric, colorimetric, titrimetric, and gasometric, that have been used in the various reduction methods for measuring the amount of reduction product formed are too numerous to discuss. Those

that have especial application to clinical procedures are exemplified in the specific methods described below.

Sugar in Urine

For this determination the copper titration method of Benedict (4, 5), which is at present in almost universal use in this country will be described. We also give the application to urine of the Shaffer-Hartmann copper titration for those who prefer this elegant iodometric procedure, the Hawkins-Van Slyke ferricyanide timing method for its extreme simplicity, and the gasometric ferricyanide method for its special advantages. The technique for determination of fermentable sugar is given for use in cases where there is reason to doubt the identity of reducing substances detected in the urine, or where it is desirable to determine accurately the glucose in urines containing only small amounts.

Non-fermentable substances in the urine, creatinine, uric acid, and others, frequently exert as much reducing effect as 0.1 or 0.2 per cent of glucose; in concentrated urines these substances may exert a reducing effect equal to that of 0.4 per cent of glucose. Hence in a simple urine sugar determination by reduction of cupric or ferricyanide reagents there is no object in an accuracy exceeding 1 gram of glucose per liter of urine.

BENEDICT'S COPPER TITRATION METHOD (5)

Benedict uses a copper reagent which is changed from Fehling's solution in such a way that all the components can be mixed without producing an unstable reagent. This advantage is gained by replacing the sodium hydroxide of Fehling's solution with the less caustic sodium carbonate, and the tartrate of Fehling's solution with citrate. Furthermore by addition of sulfocyanate Benedict achieved the end that had escaped other chemists for decades, a solution which gives a sharp end point when titrated directly by addition of sugar solution to the boiling reagent. The cuprous copper formed by reduction is precipitated, not as the red oxide, but as white cuprous sulfocyanate.

The technique of analysis consists merely of running the urine from a burette into the boiling reagent until its blue color is completely replaced by the white of cuprous sulfocyanate precipitate. The simplicity of the procedure and the calculation, and the reliability of the results have won this method its almost universal use.

Reagents

Alkaline copper solution. With the aid of heat dissolve 200 grams of crystalline sodium carbonate (or 75 grams of the anhydrous salt), 200 grams

of sodium or potassium citrate, and 125 grams of potassium thiocyanate in enough water to make about 800 cc. of solution. Filter. Dissolve exactly 18.0 grams of pure crystalline copper sulfate ($CuSO_4 \cdot 5\ H_2O$) separately in about 100 cc. of water and pour this solution slowly into the first liquid, stirring constantly. To the resulting mixture add 5 cc. of a 5 per cent solution of potassium ferrocyanide. Cool and dilute the final solution to exactly 1 liter. Of the various constituents only the copper sulfate need be exactly weighed. Twenty-five cubic centimeters of the reagent are reduced by 50 mg. of glucose. The reagent should be standardized by titration against a known solution of pure glucose by the regular procedure described below for urine analysis. If any red precipitate forms in the course of the titration the solution is unsatisfactory. Ferrocyanide obtained on the market is somewhat variable. If the original directions of Benedict which are given below are followed, occasionally a reagent will be obtained in which the reduced copper oxide is precipitated. This is usually due to an insufficiency of the ferrocyanide and can be overcome by the addition of a small excess of the salt.

Crystalline sodium carbonate.

Powered pumice or talcum.

Procedure

Before the titration the urine is diluted, if necessary, so that it contains not more than 1 per cent of sugar. Diabetic urine is ordinarily diluted 10-fold.

To 25 cc. of the reagent in a wide-mouthed Erlenmeyer flask add about 15 grams of crystalline sodium carbonate (or half as much of the anhydrous salt), and a small amount of powdered pumice stone or talcum. The mixture is heated to boiling over a free flame and should be kept vigorously boiling throughout the titration. As soon as the carbonate has completely dissolved, the diluted urine is admitted, rapidly at first, until a chalk-white precipitate forms and the blue color of the mixture begins to fade perceptibly. It is then run in a few drops at a time until the last trace of blue disappears from the solution. Half-minute intervals must be allowed to elapse between additions of successive portions of urine in the final steps of the titration. If the mixture becomes too concentrated during the titration, water may be added from time to time to replace the volume lost by evaporation. The end-point must be determined while the solution is still hot. If it is allowed to cool the reaction tends to undergo a certain amount of reversal and the solution will reassume a slight bluish-green tint. With pure glucose

solutions the final mixture is entirely colorless except for the grayish appearance that may be imparted to it by the pumice. With urine a slight yellowish green color due to the urinary pigments remains even after the copper has been entirely reduced.

Calculation

The titration measures the volume of urine which contains 50 mg. of glucose. Hence

$$\frac{50}{V} = \text{grams of glucose in 1 liter of urine.}$$

V = the number of cubic centimeters of undiluted urine used in the titration.

The method is useful only if the urine contains as much as 0.2 per cent of sugar.

BENEDICT'S METHOD FOR QUALITATIVE DETECTION OF SUGAR IN URINE (5A)

Benedict's test for sugar in urine is so much used in following diabetic cases, that it has seemed desirable to insert it here.

Reagent

There is but one solution. It is made as follows:

Copper sulfate, crystallized	17.3 grams
Sodium citrate	173.0 grams
Sodium carbonate, anhydrous	100.0 grams
Distilled water	to 1000.0 cc.

With the aid of heat dissolve the sodium citrate and carbonate in about 600 cc. of water. Pour (through a folded filter if the solution is not clear) into a 1 liter graduate and make up to 850 cc. Dissolve the copper sulfate in about 100 cc. of water and make up to 150 cc. Pour the carbonate-citrate solution into a large beaker or casserole and add the copper sulfate solution slowly, with constant stirring.

Procedure

Place 5 cc. of the reagent in a test tube. Add 8 drops (not more) of urine. Heat to boil and keep boiling 1 to 2 minutes, then let cool ***spontaneously.*** In the presence of more than 0.08 per cent of glucose the ***entire solution*** becomes filled with a precipitate, which may be red, yellow, or green. If the amount of glucose is small, the precipitate forms only on cooling. If none is present, the solution remains clear, or a faint turbidity due to urates may appear. Sometimes it is difficult to tell whether the precipitate is characteristic of glucose or not. In such a case a quantitative fermentation test (see p. 452) is necessary.

SHAFFER AND HARTMANN'S COPPER TITRATION METHOD (37)

An alkaline citrate-oxalate-$CuSO_4$ solution containing a known amount of KIO_3 and excess KI is heated with the sugar, then at once acidified. The iodate sets free an equivalent of I_2 by the reaction, $5\ HI + HIO_3 = 3H_2O + 3\ I_2$. Of the I_2 set free, part is at once reduced again by the cuprous copper which has been formed by the action of the sugar. $2\ Cu^+ + I_2 = 2\ Cu^{++} + 2\ I^-$. The decrease in titratable iodine therefore measures the sugar. The oxalate combines with the Cu^{++} to form non-ionized cupric oxalate, so that the reaction goes completely from left to right. The end-point is somewhat sharper than in Benedict's method.

Reagents

Only potassium salts may be used in this method. Sodium salts in equivalent amounts can not be substituted.

Alkaline copper-iodide reagent. Dissolve 81 grams of crystalline potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$), 70 grams of anhydrous potassium carbonate and 92 grams of crystalline potassium oxalate ($K_2C_2O_4 \cdot H_2O$) in about 600 cc. of warm water. Into this solution pour, through a funnel extending to the bottom of the beaker, with constant stirring, 25 grams of crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$) which has been dissolved separately in a small amount of water. Dissolve 3.57 grams of potassium iodate and 50 grams of potassium iodide in 150 to 200 cc. of warm water and add this to the alkaline copper solution. Cool and dilute the final mixture to 1000 cc.

5.0 N sulfuric acid solution (approximate). Dilute 140 cc. of concentrated sulfuric acid (specific gravity 1.835 to 1.849) to a liter.

0.1 N potassium thiosulfate solution, standardized by titration against iodate or biiodate in the presence of potassium iodide and free sulfuric acid, as described on page 33. This solution keeps better if it is protected against decomposition by CO_2 by the addition of enough sodium hydroxide to give about 0.02 N of NaOH.

Starch indicator solution, prepared as described on page 34.

Procedure

To 50 cc. of the alkaline copper-iodide reagent in a 300-cc. Erlenmeyer flask, containing one or two glass beads or bits of broken porcelain, add an amount of urine containing between 50 and 150 mg. (at any rate less than 150 mg.) glucose and enough water to bring the total volume of solution to 100 cc. (e. g., if 15 cc. of urine is taken, 35 cc. of water must be added). Cover the mouth of the flask with a small inverted beaker and place it on an asbestos mat over a flame so adjusted that the solu-

tion will come to a boil in exactly four minutes.[1] When boiling has continued exactly five minutes remove the flask from the flame and cool the contents rapidly under running water. Then add 20 cc. of 5.0 N sulfuric acid slowly, shaking the flask cautiously all the while to prevent too rapid effervescence of CO_2. Solution of the cuprous oxide may be aided by warming the flask slightly (to about 40°). Titrate with 0.1 N potassium thiosulfate until the green color is almost entirely dispelled. At this point add 1 cc. of soluble starch indicator solution and complete the titration.

Calculation

A blank analysis of the reagents gives the volume, B, of thiosulfate required to titrate the I_2 from all the iodate in the reagent. In the sugar analysis a smaller volume, A, of thiosulfate suffices to titrate the I_2 left after part has been reduced by the Cu^+ formed from Cu^{++} by the sugar. The sugar is proportional to the difference, $B - A$. One cubic centimeter of thiosulfate should be equivalent to 6.36 mg. of copper or 2.92 mg. of glucose. The calculation therefore is according to the formula

$$\text{Grams of glucose in sample} = 0.00292\ (B - A)$$

or

$$\text{Grams of glucose in 1 liter urine} = \frac{2.92\ (B - A)}{V}$$

B and A = cubic centimeters thiosulfate to titrate blank and unknown, respectively. V is the cubic centimeters of urine in the sample.

Because slight variations in the conditions under which heating is carried out may alter the factor from 2.92 it is well to test reagents with known glucose solutions. An empirical curve may be constructed for each new batch of copper reagent by the titration of samples containing 25, 50, and 100 mg. of glucose. Such a curve permits the immediate translation of cubic centimeters of 0.1 N thiosulfate into terms of glucose. The thiosulfate itself must, however, be standardized at intervals against iodate or biiodate.

Precautions. The volumes of the various solutions and the time consumed in boiling and cooling must not be altered in the least if satisfactory quantitative results are desired. If all the details of technique are meticulously observed the method is susceptible of extreme accuracy.

VAN SLYKE AND HAWKINS' (42) GASOMETRIC FERRICYANIDE METHOD

This method is described in the section on gasometric methods, page 472.

[1] This is not hard to effect with a micro burner if the latter is shielded from air currents.

HAWKINS AND VAN SLYKE'S TIMING FERRICYANIDE METHOD (24, 25)

The principles of this procedure and the reagents and apparatus required for it are the same as those used for the analysis of blood, which is described on page 475. The method is planned for use with urines containing so much glucose that measurement of total reducing substances gives sufficient information. It is accurate to within 1 gram of glucose per liter of urine.

Reagents

Ferricyanide solution. Same as for blood sugar, page 475.

Procedure

Dilution of urine. Ordinarily 1 cc. of urine is diluted with water to 100 cc. In urine so diluted the ferricyanide reagent will determine up to 3 per cent of glucose. In case the sugar content is known to be above 2 per cent, 1 cc. of urine is diluted to 200 or 400 cc., so that glucose up to 6 and 12 per cent respectively can be determined. On the other hand, if the urine as voided is presumably of low sugar content, it is best to dilute only 25-fold. Dilution is the only preliminary treatment of the urine required, as even albumin does not affect the determination.

Decolorization of ferricyanide by diluted urine. 0.5 cc. of diluted urine is pipetted into a thin-wall Pyrex test-tube (9 by 90 mm. outside measurement) followed by 0.5 cc. of ferricyanide solution. The procedure from this point is exactly the same as in the blood sugar method described on p. 475.

The amount of sugar in the urine is found by use of the curve in figure 63. If the urine has been diluted 200-fold instead of 100-fold, the sugar concentrations indicated by the curve are doubled. On the other hand, they are halved if the dilution has been only 50-fold.

APPLICATION OF BLOOD SUGAR METHODS TO THE ANALYSIS OF URINE

Most of the methods which have been described for the analysis of blood may be applied to the analysis of urine, if the latter is sufficiently diluted and its color removed by some means. For the latter purpose various agents have been employed.

The results obtained by these delicate blood sugar methods on normal urine or urine with only "traces" of sugar are, of course, grossly erroneous, because of the large amounts of non-glucose reducing substances present. In such urines greater accuracy can be obtained if these substances are

partly removed. For this purpose the Patein-Dufau mercury precipitation used by Benedict and Osterberg (10) is probably as satisfactory as any agent. The only accurate method as yet available, however, is rapid yeast fermentation.

DETERMINATION OF FERMENTABLE SUGAR IN URINE BY MEASURING THE DECREASE IN REDUCING SUBSTANCES CAUSED BY TREATMENT WITH YEAST. VAN SLYKE AND HAWKINS (43)

This procedure, with minimum dilution of the urine, is designed primarily for urines containing amounts of reducing substances of the order of magnitude found in non-diabetic cases. It is usually only in urines with slight reducing powers that one needs to determine fermentability of the material. The method as given is designed for urines with reducing powers not exceeding that of a 0.5 per cent glucose solution. Urines more heavily loaded with reducing substances must be diluted sufficiently to bring the total reducing power below that of a 0.5 per cent glucose solution.

Reagents

Oxalic acid solution, 0.1 M. Dissolve 9.0 grams of oxalic acid in water and dilute it to 1 liter.

Lloyd's alkaloidal reagent, a preparation of fuller's earth.

Yeast suspension. Twenty grams of Fleischmann's compressed yeast cake are suspended in 50 cc. of water. It is unnecessary to wash the yeast because there is so little reducing material in yeast compared with that in urine. The suspension contains about 40 volumes per cent of yeast, as determined by centrifugation.

Procedure

Preparation of urine for analysis. The urine is subjected to preliminary treatment with Lloyd's reagent to remove creatinine, uric acid and certain other non-fermentable reducing substances. The substitution of oxalic acid for the sulfuric acid used by Folin and Berglund (16a) obviates the formation of a calcium precipitate if the urine is later to be mixed with ferricyanide-carbonate solution.

A. To 10 cc. of urine add 5 cc. of 0.1 N oxalic acid, 5 cc. of water and 1.5 grams of Lloyd's reagent. Shake the mixture gently for two minutes and then filter.

B. A control filtrate is made at the same time using water instead of urine.

C. To another 10 cc. of urine are added 7.5 cc. of yeast suspension. The mixture is allowed to stand for fifteen minutes. Five cubic centi-

meters of 0.1 N oxalic acid and 1.5 grams of Lloyd's reagent are then added. After the mixture has been shaken for two minutes it is filtered. Under these conditions the yeast will remove glucose in amounts not exceeding 0.5 per cent.

D. A control filtrate is prepared from a mixture in which 10 cc. of water instead of 10 cc. of urine are treated as in *C.*

Analysis of the 4 filtrates for sugar can be carried out by the gasometric urine sugar method (p. 408) or by any one of the methods described below for the determination of sugar in blood filtrates.

Calculation

$$(A - B) - (C - D) = \text{fermentable urine sugar.}$$

Blood Sugar

TYPES OF REDUCTION METHODS USED AND SIGNIFICANCE OF RESULTS

Until the last decade studies of the blood sugar were largely confined to the physiological laboratory because of the lack of suitable methods. Bertrand's (11) method, presented in 1906, met with little immediate response from clinicians because it required more blood than could be readily obtained from a small incision of the skin. When venous puncture became a common diagnostic procedure, a few years later, sporadic studies of the blood sugar appeared from various clinics. The total sum of these studies, however, was small before 1913. In that year appeared the micro-copper method of Bang (2) and the picrate method of Lewis and Benedict (30, 31), which permitted the determination of sugar on small amounts of blood, and Bang (3) published his treatise on the blood sugar. There was an immediate response on the part of both chemists and clinicians that has led to the appearance of numerous micro methods and an enormous amount of work in the study of blood sugar.

All the clinically applicable procedures thus far proposed take advantage of a single property of glucose, its activity as a reducing agent in hot alkaline solution. This, as stated already, is a property which is not specific for glucose. It is shared by many other substances that are found in appreciable concentration in normal blood, including creatinine, uric acid, glutathione, and ergothionine. According to Somogyi (39) the last two represent most of the non-glucose reducing material. In general it appears that the method which indicates most nearly the true glucose content of blood is that one which will recover glucose quantitatively in pure solution and when it is added to blood, and which, at the same time, gives lowest values when applied to blood.

The methods in use before the copper procedures of Benedict (7, 9) appeared in 1926 and 1928 were fairly uniform in indicating the total reducing substances of the blood of normal fasting subjects to be usually 100 to 110 mg. per 100 cc., with occasional variations of 20 or even 30 mg. outside this range. Benedict's blood sugar reagents, however, give about 20 mg. less than previous methods. The recent colorimetric ferricyanide method of Folin (15, 16), likewise gives results averaging about 20 mg. less than those by other methods in the literature. Both these methods accurately indicate known amounts of glucose added to either water or blood. From these facts it seems that these two procedures are less influenced by non-glucose substances in the blood then other methods, and give more nearly true glucose values. Herbert, Bourne, and Groen (26) find that the Benedict reagent (9) is unaffected by glutathione, which reduces most other reagents. These authors also present evidence that glutathione or other cell constituents are responsible for previous methods' giving higher results than Benedict's (9). They find that when the blood proteins are precipitated (as described on p. 67) without laking the cells, analyses of the filtrate by three methods which ordinarily show higher sugar values give the same results as analysis by Benedict's method (9). Criticisms of Everett (13), that the color developed by Benedict's reagent with blood filtrates fades enough more rapidly than that developed with standard glucose solutions to invalidate quantitative comparison, have been effectively answered by Benedict (8). However, even the Benedict reagent does not appear to give reduction values due to glucose and nothing else. After fermentation blood filtrates still show with this reagent reductions equivalent to 6 to 14 mg. of glucose per 100 cc. of blood (9, 43). Also it appears possible that it may fall a little short of giving 100 per cent reduction with the glucose that is present. Van Slyke and Hawkins (43) found that the Benedict reagent gave about 10 mg. lower values for fermentable sugar than other (37, 42) methods, and Benedict (9), when he added known amounts of glucose to previously fermented blood, found by his method increases of 85 to 93, instead of 100 per cent, of the amounts added.

Apparently the following conclusions are justified. None of the methods at present available indicates exactly the glucose of the blood. Most methods except the latest ones of Benedict (7, 9) and Folin (15, 16) show in analyses of filtrates from whole blood amounts of reduction due to non-fermentable substances equivalent to the reduction caused by 20 to 30 mg. of glucose per 100 cc. of blood. The recent Benedict (9, 9a), and Folin (15, 16) methods show less non-fermentable reducing substances and also less fermentable, and the results are probably nearest to the true glucose values.

However, any of the standard procedures may be trusted ordinarily to show whether a blood sugar is normal or how much it deviates therefrom. In interpreting results the important consideration is that the Benedict (9) and recent Folin (15, 16) procedures show about 20 mg. less "sugar" per 100 cc. of whole blood than other methods. It is by the other methods that practically all clinical data now in the literature have been obtained, on which the discussion of blood sugar in volume I is based.

The relative values given by the Benedict (9) and Folin (15, 16) methods, and by the Shaffer titration and the gasometric ferricyanide procedures, which agree approximately with most methods other than the Benedict and Folin, are exemplified in table 50.

TABLE 50

Comparison of Blood Sugar Values by Different Methods. Data Include Both Normal and Glycemic Bloods. From Van Slyke and Hawkins (43)

Method	Milligrams of sugar per 100 cc. blood compared with that by Benedict's (9) method taken as 100		
	Average	Maximum	Minimum
Shaffer-Hartmann-Somogyi copper titration (38)	121	149	109
Folin ferricyanide colorimetric (15, 16)	98	124	86
Van Slyke-Hawkins ferricyanide gasometric and timing methods (24, 43)	120	138	115

Choice of methods

The six blood sugar methods described in this volume are the copper titration of Shaffer, Hartmann, and Somogyi, the ferricyanide titration of Hagedorn and Jensen, the colorimetric copper method of Benedict, the colorimetric ferricyanide method of Folin, the gasometric ferricyanide method of Van Slyke and Hawkins, and the timing ferricyanide method of the same authors. All of these methods are rapid, and all yield consistent, although as stated above, not identical results.

If one is limited to blood samples of 0.1 cc. the choice of methods is limited to the Folin colorimetric, the micro form of Benedict's colorimetric, and the Hagedorn-Jensen titration. If 0.2 cc. blood samples are regularly available, or aliquot parts of the Folin-Wu filtrate prepared for general blood analysis, any of the six methods may be used.

Between the two titration methods, the choice is largely one of the type of filtrate on which it is convenient to work. Both titrations are beautiful

iodometric procedures. The Shaffer is applied to the tungstic acid filtrate, and is most convenient when for general purposes that filtrate is routinely prepared. The Hagedorn-Jensen involves preparing by a rapid method its own filtrate from 0.1 cc. of blood by zinc deproteinization, and has with other ferricyanide methods the advantage of immunity from reoxidation by atmospheric oxygen. It is so convenient that it has acquired almost universal use in Europe.[2]

Of the two colorimetric methods it is difficult to say by way of comparison more than that the analyst may follow his artistic preference for colors and ease of manipulation.

For one who has and enjoys the use of the manometric gas apparatus, the gasometric ferricyanide method offers its advantages of objective accuracy of measurement and freedom from standard solutions.

The timing method, which is based on measurement of the number of seconds required to decolorize a yellow ferricyanide solution, is with respect to reagents, apparatus, and manipulation, the simplest of all, and permits performance of the most determinations in a given time. It is practically a bedside method. The error of the timing procedure, about ± 5 per cent, is not sufficient to affect the clinical interpretation of results.

In the use of all blood sugar methods it is necessary to observe the precautions mentioned in chapter 2 to prevent loss of sugar by glycolysis after the blood is drawn.

COLORIMETRIC COPPER METHOD OF BENEDICT (9a)[3]

This method utilizes a procedure introduced by Folin and Wu (20) in which the cuprous salt formed by reduction is permitted to react with a molybdate solution The molybdate is partially reduced by the Cu^+ to lower oxidation products of blue color, the intensity of which is a measure of the amount of copper reduced to the cuprous condition, and therefore of the sugar.

While Folin himself has abandoned this reagent in favor of the one next described, based on ferricyanide reduction, Benedict (6, 7, 8, 9, 9-*a*) has studied

[2] For additional volumetric procedures see: Forschbach and Leverin, Arch. f. exp., Path. Pharm., 1912, 68, 341; Michaelis, L., Biochem. Z., 1914, 59, 166; Kowarsky, A., Deutsch, med. Woch., 1919, 45, 188; Schirckauer, Hans, Berl. klin. Woch., 1920, 57, 227; Svend, Schweiz, med. Woch., May 5, 1921; Kleiner, I. S., J. Am. Med. Ass., 1921, 76, 172.

[3] Kiefer (29) has devised convenient portable apparatus which permits the determination of blood sugar by the Folin and Wu method at the bedside of the patient with sufficient accuracy for most clinical purposes. He employs dilution colorimetry with a comparator. The apparatus can be equally well used for the Benedict technique.

to improve the copper-molybdate reagent, making it more specific and sensitive. Benedict's final reagent (9a) is a modification of Fehling's, in which the alkalinity is diminished by substituting sodium carbonate for hydroxide, and alanine is added because the amino acid appears to form with cupric salts a complex which is relatively unaffected by the non-glucose substances of the blood that reduce other copper reagents The addition of sodium sulfite accentuates this effect, increasing the reactivity of the Cu^{++} with glucose and making the reactivity relatively less with non-glucose substance in the blood filtrate. In consequence the latest Benedict reagent, applied directly to tungstate or tungstomolybdate blood filtrates, determines nonfermentable substances equivalent in reducing power to only 4 to 8 mg. of glucose per 100 cc. of blood.

Reagents

Copper reagent. This reagent has the following composition:

	grams
Sodium carbonate, anhydrous	15
Alanine	3
Rochelle salt	2
Copper sulfate, crystallized	3
Distilled water to make 500 cc.	

The alanine, Rochelle salt, and copper sulfate should be weighed accurately. The sodum carbonate may be weighed more roughly. Dissolve the carbonate, alanine, and Rochelle salt in 300 to 400 cc. of distilled water. Dissolve the copper sulfate separately in 50 to 75 cc. of distilled water, and add this to the other solution with constant stirring. Dilute to 500 cc. and mix. If kept in an ice box this solution will remain without appreciable deterioration for six to eight weeks. In a warm room it deteriorates more rapidly, as indicated by increase in the blank.

One per cent sodium bisulfite solution.

Copper bisulfite reagent. To the copper reagent add 1/20th its volume of the 1 per cent sodium bisulfite solution. Only enough of the mixture is prepared for the day's analyses, as it deteriorates after one or two days.

Phosphomolybdic acid color reagent. Place 150 grams of pure molybdic oxide (which must be free of ammonia) in a large Erlenmeyer flask, and add 75 grams of pure anhydrous sodium carbonate. Add 500 cc. of water in small portions; shaking after each addition. Shake thoroughly and heat to boiling or until nearly all of the molybdic oxide has been dissolved. An appreciable amount of insoluble material remains, which is filtered off. The residue on the filter is washed with water until the total volume of filtrate

and washings is about 600 cc. Add 300 cc. of 85 per cent phosphoric acid (the concentrated, syrupy phosphoric acid of specific gravity 1.72) to the total filtrate, cool, and dilute to 1 liter. This solution has a slight yellow color when viewed in large volumes, but it does not affect significantly the blue color developed when the solution reacts with cuprous oxide.

Tungstic acid reagents of Folin and Wu or tungstomolybdic acid reagent of Benedict for precipitating blood proteins. These are described on pages 65 and 68 respectively.

Standard glucose solutions. A stock standard 1 per cent glucose solution is prepared by dissolving 1 gram of pure, anhydrous glucose in water and diluting to 100 cc. Glucose deteriorates rapidly in alkaline solution and is also destroyed by bacteria, yeasts and molds. To prevent these changes Benedict (6) recommends the addition to the 1 per cent aqueous solution of a few cubic centimeters of toluol. As a further precaution the solution should be kept in the refrigerator. Under any circumstances it must be tested from time to time.

Dilute blood sugar standards. Dilute 1 part of the stock standard to 100 with water, to make a standard containing 0.1 mg. of glucose per cubic centimeter, and layer the solution with a little toluol. This standard must be renewed at least once a week. If the level of the blood sugar is above 160 to 200 mg. or below 75 to 50 mg. per 100 cc. standards twice as strong or half as strong must be used, unless Rothberg and Evans tubes are employed (see below). These tubes permit the use of a single standard containing 0.1 gram glucose per liter for the analysis of all bloods containing not more than 400 or less than 50 mg. of glucose per 100 cc.

Special blood sugar tubes. The special Folin and Wu blood sugar tube is shown in figure 62. It is calibrated at 25 cc. and has a constriction above a bulb at the bottom. The constricted portion of the tube must not be more than 8 mm. in diameter and the bulb at the lower end must be of such a size that when 4 cc. of fluid are placed in the tube the upper level of the fluids will lie in the constricted portion. Rothberg and Evans (35) have suggested that the tubes for the standard sugar solutions be calibrated at 12.5 and 25.0 cc. and that the tubes for the unknown solutions be calibrated at 2.5 cc. intervals from 12.5 to 50.0 cc. By varying the dilutions standard and unknown can be so adjusted that specimens of blood containing as little as 50 or as much as 400 mg. per cent of glucose can be analyzed with a single standard containing 0.1 gram of glucose per liter. The use of tubes marked at 25 cc. with two standards, containing 0.1 and 0.2 gram of glucose per liter meets all ordinary requirements. For the micro procedure described below special modified Rothberg and Evans tubes are employed by the authors. These are graduated at 1.25 cc. intervals from 6.25 to 25.00 cc. The con-

stricted portion of the tube has an internal diameter of only 8 mm. and the bulb holds only 1 cc.

In place of the specially constricted Folin-Wu or Rothberg and Evans tubes ordinary tubes properly graduated may be used, according to Benedict (6), with the following precautions to prevent reoxidation of the cuprous oxide. After the solutions are placed in the tubes and before they are heated, 3 to 4 drops of benzene are added and the tubes are stoppered with cotton. During the heating the benzene is completely vaporized and the

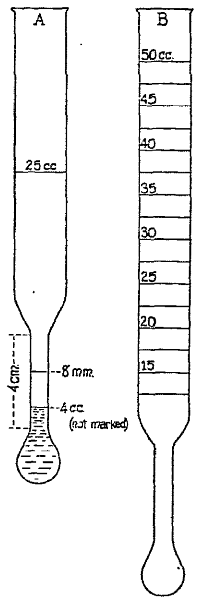


FIG. 62. *A*, Folin-Wu blood sugar tube; *B*, Rothberg-Evans tube. The purpose of the constriction is to diminish the surface of solution exposed + atmospheric oxygen.

heavy vapor remains in the tube, excluding the air. Shaffer (personal communication) prefers to cover the mouths of the tubes with glass bulbs. These become sealed to the mouths of the tubes, shortly after heating is begun, by condensed water vapor. Most of the air is driven from the tubes by the heat, and convection currents in the remaining air are prevented by the sealed bulb.

Procedure

Transfer 2 cc. of Folin Wu or Benedict 1 to 10 blood filtrate (see precipitation of blood proteins, p. 65 and 68) to a Folin and Wu or Roth-

berg and Evans blood sugar tube, and to another similar tube an equal amount of standard glucose solution. To each add 2 cc. of the copper-bisulfite reagent. Mix the contents of the tubes by shaking laterally and then place the tubes in boiling water for five and one-half to six minutes. At the end of this time cool them by immersion in cold water. Add 2 cc. of the color reagent. Mix the solutions. The cuprous oxide dissolves at once. After one to two minutes dilute the standard to 25 cc. and the unknown to the same volume or, in case a Rothberg-Evans tube is used, to the volume which gives an approximate color match with the standard. The reading in the colorimeter is preferably made within 10 minutes after dilution.

Precautions. Care must be taken to see that all the reduced copper is dissolved by the phosphomolybdic acid reagent before the solution is diluted. Colorimetric readings must be made as soon as possible after the colors have been developed, because the colors tend to change after a certain time (8, 13). Such change does not occur for at least 10 minutes. No more solutions should be heated together than can be read in the colorimeter within 10 minutes. The directions for heating and cooling the solutions must be followed with scrupulous care because variations in temperature or time affect the results significantly.

Calculations

$$\frac{1000\ S\ V_u\ n}{U\ V_s} = \text{milligrams of glucose in 100 cc. of blood.}$$

S and U = colorimetric readings of standard and unknown respectively; V_s and V_u = the cubic centimeters to which standard and unknown, respectively, were finally diluted; n = the strength of the standard solution in milligrams of glucose per cubic centimeter.

When, as in usual routine analyses $V_s = V_u$ and $n = 0.1$, the formula simplifies to:

$$\frac{100\ S}{U} = \text{milligrams of glucose in 100 cc. of blood.}$$

MICRO FORM OF BENEDICT'S COLORIMETRIC METHOD (9a)

The reagents are the same as for the macro method described above, except for the tungstic acid or tungstomolybdic acid solution used to precipitate the blood proteins.

Tungstomolybdic acid solution. Of the tungstomolybdate solution, described on page 68, 5 cc. are measured into a 250-cc. volumetric flask. About 150 cc. of distilled water are added, followed by 5 cc. of the 0.62 N sulfuric acid. The solution is diluted to 250 cc. and mixed. It should be prepared fresh within three to five days of its use. This is the solution used by Benedict (9a).

Tungstic acid solution. Twenty cubic centimeters of the 10 per cent sodium tungstate solution, described on page 65, are measured into a 500-cc. volumetric flask and diluted to about 400 cc. Twenty cubic centimeters of the $\frac{2}{3}$ N sulfuric acid are then added, and the solution is made up to 500 cc. The solution is kept protected from light, and is renewed whenever an appreciable precipitate appears in the bottom, or any trace of blue color is evident.

Copper-bisulfite reagent. To the copper reagent described for the macro method 0.1 its volume of the 1 per cent bisulfite is added. Only enough of the mixture is prepared for the day's analyses, for it deteriorates after one or two days.

Standard glucose solution, 0.002 per cent. One cubic centimeter of the stock 1 per cent solution is diluted to 500 cc.

Procedure

For precipitation of the blood measure 5 cc. of either the tungstomolybdic acid or tungstic acid solution into a 15-cc. centifuge tube. In a pipette calibrated to ***contain*** 0.1 cc. measure 0.1 cc. of blood and discharge this vigorously into the solution in the centrifuge tube, and rinse the pipette 2 or 3 times with portions of the mixture. Stopper the centrifuge tube, shake vigorously, and, after a minute or longer, centrifuge for three minutes.

Measure 2 cc. of the clear supernatant blood filtrate into a Folin-Wu sugar tube, add 1 cc. of the copper-bisulfite reagent, and mix. Add enough benzene to fill the constricted portion of the tube.

In a similar tube one measures 2 cc. of the 0.002 per cent glucose standard solution, 1 cc. of the copper-bisulfite reagent, and benzene.

Both tubes are heated for exactly five minutes by immersion in a vigorously boiling water bath. They are then cooled for one minute in a large volume of cold water (avoid shaking, which would favor absorption of air and reoxidation of reduced Cu^+). Two cubic centimeters of the color reagent are added, the solutions are mixed by lateral shaking, and 3 cc. of water added to each tube. The contents are mixed at once by inversion, and read in the colorimeter within ten minutes.

Calculation

$$\frac{102\ S}{U} = \text{milligrams of glucose per 100 cc. of blood.}$$

In case the sugar content is so high that S/U exceeds 2, the determination may be repeated with only 1 cc. of blood filtrate plus 1 cc. of water in place of 2 cc. of filtrate. The calculation then is

$$\frac{204\ S}{U} = \text{milligrams of glucose per 100 cc. of blood.}$$

COLORIMETRIC FERRICYANIDE METHOD OF FOLIN (15, 16, 17)

The sugar is oxidized with alkaline potassium ferricyanide and the ferrocyanide produced is measured colorimetrically as Prussian blue. Gum ghatti is added to keep the Prussian blue in colloidal solution. So intense is the resulting color that two separate determinations can be made on the extract from 0.1 cc. of normal blood.

The reactions are:

$$\underset{\substack{\text{ferricyanide}\\ \text{(yellow)}}}{K_3Fe(CN)_6} + \text{Sugar} \longrightarrow \underset{\substack{\text{ferrocyanide}\\ \text{(colorless)}}}{K_4Fe(CN)_6}$$

$$\underset{\text{colorless}}{3\ K_4Fe(CN)_6} + \underset{\text{yellow}}{2\ Fe_2(SO_4)_3} = \underset{\text{Prussian blue}}{Fe_4(Fe(CN)_6)_3} + 6\ K_2SO_4$$

The final color is a mixture of the yellows of the excess ferricyanide and ferric sulfate with the blue of the Prussian blue. To eliminate the effect of the yellows, a light filter is used.

Reagents

Dilute tungstic acid. Transfer 20 cc. of 10 per cent sodium tungstate, prepared as described for the Folin-Wu precipitation of proteins (p. 65), to a 1-liter volumetric flask and dilute to about 800 cc. Add, while shaking, 20 cc. of $\frac{2}{3}$ N sulfuric acid and dilute to the mark. The acid must be accurately prepared. The reagent should be kept protected from the light. From time to time it should be tested for reducing materials by a blank on 2 cc. of reagent + 2 cc. of water. The reagent must be discarded if any blue color is obtained in the blank test. When the reagent stands longer than a fortnight a precipitate may form, with loss of the power to precipitate proteins.

Potassium ferricyanide. Dissolve 2 grams of potassium ferricyanide[4]

[4] Potassium ferricyanide should be almost completely free from ferrocyanide and should always be tested for purity in this respect in the following manner. To 2 cc. of a 1 per cent solution of ferricyanide in a test tube add 3 cc. of water and 1 cc. of ferric iron solution.

in water and dilute to 500 cc. The major part of this solution should be kept in a brown bottle in a dark closet. Reagent for daily use is also kept in a brown bottle and is stocked with only enough solution to last for 1 week.

Sodium cyanide-carbonate. Dissolve 1.5 grams of sodium cyanide in water and dilute to 150 cc. Transfer 8 grams of anhydrous sodium carbonate to a 500-cc. volumetric flask and dissolve it by means of about 50 cc. of water. With a cylinder (avoid pipette, poison!) add 150 cc. of the 1 per cent cyanide solution. Dilute the mixture to 500 cc. and mix. Discard the remainder of the cyanide solution.

Ferric iron. Fill a liter cylinder to the mark with cold water. Push into this cylinder a circular piece of galvanized iron or copper window screening, large enough to form a bowl well below the surface of the water. On the screening place 20 grams of gum ghatti.[5] Cover the cylinder and set it aside for eighteen to twenty-four hours. At the end of this time remove the screen containing the undissolved residue, and strain the liquid through a double layer of clean towelling to remove dirt which has passed through the screen.

By the aid of heat dissolve 5 gram of anhydrous ferric sulfate with 75 cc. of syrupy (85 per cent) phosphoric acid and 100 cc. of water. Cool the solution and mix it with the gum ghatti solution.

To the final mixture add 1 per cent potassium permanganate solution, at first 5 cc., and later about 3 cc., at a time, until the pink color of permanganate remains perceptible for at least five to ten minutes. This step is essential for the oxidation of certain materials in the gum ghatti which reduce ferricyanide.

If there is more than a trace of blue color at the end of 5 minutes, the salt must be recrystallized by the following process:

In a liter beaker set in warm water (50°) dissolve 100 grams of potassium ferricyanide in 400 cc. of water by stirring. When the salt is dissolved filter the solution into a flask through a 24 cm. filter which has been previously washed with water. Cover the mouth of the flask with a beaker and cool the filtrate under running water. To 600 cc. of alcohol in a flask add about 0.1 cc. of bromine, and shake the solution a moment. Transfer the cold ferricyanide solution to a 2 liter beaker and add the brominated alcohol, with stirring. The ferricyanide comes down at once. The solution is filtered immediately through a well fitting hardened filter paper on a 10-cm. Buchner funnel. The precipitate is washed on the filter with 150 cc. of alcohol to which 2 or 3 drops of bromine have been added, then with 100 cc. of ether containing enough bromine to give it a good straw-yellow color, and finally with 25 cc. of ether containing no bromine. The last ether is removed as completely as possible by strong suction. The precipitate is then blown on to a large watch-glass or plate and dried at about 50°. The yield should be about 80 grams.

[5] Gum ghatti no. 2, suitable for this purpose can be purchased from Howe and French, in Boston, or from Eimer and Amend, in New York.

Standard glucose solution. Dissolve 2 grams of benzoic acid in about 500 cc. of hot water in 1-liter Florence flask. With the aid of the warm benzoic acid solution rinse 2.000 grams of pure anhydrous glucose into a liter volumetric flask. Add water to make about 900 cc., cool to room temperature, dilute to volume and mix. Keep this stock standard solution, which contains 2 mg. of glucose per cubic centimeter, in a glass-stoppered bottle.

From the stock solution the dilute working standard, containing 0.01 mg. of glucose per cubic centimeter is prepared as needed in the following manner. In a 2-liter volumetric flask place 0.5 gram of benzoic acid and about 1500 cc. of water. Add 10 cc. of the stock glucose solution and shake occasionally until the benzoic acid has dissolved. Dilute the solution to 2 liters and mix. The dilute standard should be kept covered with a thin layer of toluol in a glass stoppered bottle.

Procedure

Precipitation of proteins is carried out by a modified Folin-Wu technique. With a pipette accurately calibrated ***to contain*** 0.1 cc. transfer this amount of blood to a conical centrifuge tube containing 10 cc. of the dilute tungstic acid solution. Rinse the pipette by sucking the tungstic acid to the mark two or three times. (The outside of the pipette must, of course, be wiped clean of adherent blood before it is introduced into the acid.) Stir the mixture in the tube with a footed glass rod and then centrifuge three to five minutes.

Determination. To 4 cc. of the blood filtrate in a test tube of ordinary shape marked at 25 cc. add 2 cc. of the 0.4 per cent ferricyanide solution and 1 cc. of the cyanide-carbonate solution. At the same time treat in the same manner 4 cc. of the dilute standard glucose solution in a similar tube. Heat both tubes in boiling water for eight minutes. After cooling them in running water for one to two minutes, add to each 5 cc. of the acid ferric iron solution. This should be introduced along the side of the tube to prevent foaming. Mix by gentle shaking, dilute the contents to volume, mix again and compare standard and unknown in the colorimeter. The surfaces of the solutions are apt to be obscured by foam when the dilution is made. In this case, dilute to a little less than 25 cc., add two or three drops of alcohol to dispel the foam and then dilute exactly to the mark.

Preparation of yellow light filters. Because the final color is a combination of those of Prussian blue and yellow unreduced ferricyanide in varying proportions depending upon the amount of glucose present, colorimetric comparison can be made in the ordinary way only if standard and unknown are

very nearly the same. To obviate the necessity of preparing multiple standards Folin and Malmros (17) advocate the introduction of a yellow light filter. This can be prepared in the following manner. To 5 gram of picric acid, dissolved in 100 cc. of methyl alcohol, are added 5 cc. of 10 per cent sodium hydroxide solution. This solution is poured on to a pack of 8 or 10 filter papers, which have been laid on a level smooth mat of newspaper, until the filter papers are saturated and an excess of solution has soaked through at the bottom and flowed out at least 2 cm. from the filters on the newspaper mat. When all the liquid has evaporated and the filter papers are perfectly dry, pour over the pack an excess of a 3 per cent solution of paraffin in benzine (gasoline), then again leave the papers to dry. All of the filters should be evenly stained yellow. A heavy filter paper with good absorbing qualities (e.g., Schleicher and Schüll, no. 604) is most satisfactory. Such filters, supported by glass, may be attached to frames which fit over the light aperture of the colorimeter lamp. Such light filters can only be used for artificial light with strong illumination. To test the adequacy of the filter, place in one cup of the colorimeter 0.2 per cent potassium ferricyanide solution, in the other water. If the light filter is adequate the colors of the two fields can be made to match.

Calculation

$$\frac{100\ S}{1.01\ U} \quad \frac{99\ S}{U} = \text{milligrams of glucose per 100 cc. of blood.}$$

This formula holds if a standard solution containing 0.01 mg. of glucose per cubic centimeter is used. The factor $\frac{100}{1.01}$ is used because the original volume to which the blood is diluted is 10.1 cc. (10 cc. of tungstic acid solution +0.1 cc. of blood). If a standard twice as strong is used the milligrams of glucose per 100 cc. is $\frac{198S}{U}$.

TITRATION COPPER METHOD OF SHAFFER, HARTMAN AND SOMOGYI (37, 38)[6]

The method depends on the reduction of hot cupric salts by sugar to cuprous oxide. The latter is converted by acidification to cuprous sulfate

[6] Both the macro- and micro-determinations described differ somewhat from those which have been published. For the changes we thank Prof. P. A. Shaffer, to whom the manuscript of this chapter was submitted while it was in preparation. For his permission to present certain unpublished improvements on his own analytical methods and for valuable criticism and suggestions dealing with more general aspects of sugar determination, the authors desire to acknowledge their appreciation.

which is reoxidized to the cupric salt by a known excess of iodine liberated in the solution by the acidification of iodate and iodide. Oxalate is added to drive the reaction towards the cupric side by the formation of complex ions of alkali cupric-oxalate. The amount of iodine consumed in the oxidation is determined by titration with standard thiosulfate (see p. 449–50).

Reagents

Alkaline-copper-iodine reagents 1. Dissolve 20 grams of anhydrous sodium carbonate, 25 grams of sodium bicarbonate and 25 grams of Rochelle salt (sodium and potassium tartrate) in 600 cc. of water. Dissolve 7 grams of crystalline copper sulfate ($CuSO_4 \cdot 5\ H_2O$) separately in about 100 cc. of water. Introduce the copper solution into the carbonate-tartrate through a funnel, the tip of which rests on the bottom of the beaker, stirring the solution well during the addition to prevent loss of CO_2. Add to the mixture 10 grams of potassium iodide, 5 grams of potassium oxalate and 22 cc. of an alkaline standard normal solution of potassium biiodate. The latter solution contains per liter 32.498 grams of potassium biiodate, $KH\ (IO_3)_2$, and 83.3 cc. of 1 N potassium or sodium hydroxide. The mixed solution of cupric sulfate, iodide, and alkaline iodate is diluted to 1 liter. The advantage of adding an exact amount of accurate standard biiodate is that the reagent thus becomes a convenient and accurate means of standardizing the thiosulfate. Five cubic centimeters of the reagent, titrated without heating, requires 22.00 cc. of 0.005 N thiosulfate. This reagent is, according to Shaffer and Somogyi (unpublished) somewhat less sensitive to non-sugars than that proposed by Somogyi (38), results with which are quoted in table 50, and is more sensitive to low concentrations of sugar than that of Shaffer and Hartmann (37).

Reagent 2. Somogyi (unpublished) finds that a reagent still more sensitive to low concentrations of sugar and less sensitive to non-sugars can be prepared by the following formula, following the general method of preparation described above: Anhydrous sodium carbonate 25 grams, sodium bicarbonate 20 grams, Rochelle salt 25 grams, copper sulfate 7.5 grams, potassium iodide 5.0 grams, and 22 cc. of a normal biiodate solution (see above). At the concentration of Cu and iodide at the end-point the effect of tartrate holds the equilibrium even without oxalate. After this solution has been titrated with thiosulfate the blue starch iodide color returns after thirty minutes or so, but this is a matter of no consequence. The solution is especially well adapted to the analysis of Somogyi zinc filtrates described below.

A 1.0 N solution of sulfuric acid.

Standard sodium thiosulfate solution. A 0.1 N sodium thiosulfate solution, prepared by standardization against potassium biiodate or iodate after its reaction with an excess of potassium iodide and sulfuric acid (see p. 33). The thiosulfate keeps better if it is made up in about 0.02 N sodium hydroxide, which protects it from decomposition by CO_2. It should be standardized at least once a month against iodate or biiodate.

From this solution 0.005 N thiosulfate is prepared daily by 20-fold dilution with water containing a few drops of 10 per cent sodium hydroxide to a liter. The dilute thiosulfate is checked by titration of 5 cc. of the cop-

per reagent, which requires 22.00 cc. of the thiosulfate. If the copper reagent is protected from evaporation, this affords an extremely satisfactory means of standardization.

A 1 per cent soluble starch solution (for preparation, see p. 34)

Procedure

Add 2 to 5 cc. of Folin-Wu tungstic acid blood filtrate (see precipitation of proteins, p. 65), containing not more than 2 mg. of glucose, to 5 cc. of the copper reagent in a test tube, 25 by 200 mm., and dilute the solution to 10 cc. with water. Stand the tube, covered with a small beaker, or preferably a glass bulb, and supported in a metal rack to prevent agitation, in boiling water for fifteen minutes. At the end of this time remove it from the bath and cool it rapidly to room temperature in a pan of cold water (not by shaking it under running water). Add 5 cc. of 1.0 N sulfuric acid and, after about a minute, titrate with 0.005 N ~~potassium~~ thiosulfate until the green color is nearly dispelled. At this point add 1 cc. of soluble starch and complete the titration. During the titration the solution is stirred with a rod having a foot nearly the diameter of the test tube to insure rapid and complete mixing. For the most accurate results 25 cc. burettes calibrated in 0.05 cc. and read to 0.01 cc. are required.

Calculation

A is the number of cubic centimeters of 0.005 N thiosulfate required to titrate the unknown.

B is the cubic centimeters required to titrate the blank.

C is the cubic centimeters of 0.005 N thiosulfate equivalent to the Cu^{++} reduced to Cu^{+} by sugar.

$$C = B - A.$$

C is not exactly proportional to the amount of sugar, although it is a linear function of it. With Reagent 1 the relationship, when 5 cc. of blood filtrate are titrated, is expressed by the equation

$$\text{Milligrams of sugar per 100 cc. blood} = 21.8\,C + 16.$$

To avoid the necessity of carrying out this calculation table 51-A is given, in which values of C are translated directly into milligrams of sugar per 100 cc. of blood. Table 51-B gives similar data for Reagent 2. When the cubic centimeters of filtrate titrated are other than 5, the values in the tables are multiplied by $\dfrac{5}{\text{cubic centimeters filtrate}}$.

If preferred an empirical curve of glucose equivalents may be constructed for each new copper solution by the titration of samples containing various known quantities of glucose from 0.05 to 0.40 mg. Such a curve permits the immediate translation of cubic centimeters of thiosulfate into terms of milligram of glucose. It does not, however, eliminate the necessity of standardizing the thiosulfate.

TABLE 51-A

For Shaffer-Hartmann-Somogyi Blood Sugar Method with Reagent No. 1

Amounts of glucose (milligrams per 100 cc. of blood) corresponding to titration values (difference from blank) when 5 cc. of 1:10 blood filtrate are heated for fifteen minutes in 25 by 200 mm. covered test tubes in a boiling water bath.

Cubic centimeters of 0.005 N thiosulfate B—A	Tenths of 1 cc. of 0.005 N thiosulfate solution Milligrams of glucose in 100 cc. of blood									
0		14	16	18	21	23	25	27	30	33
1	35	37	39	42	44	46	48	50	53	55
2	58	60	62	65	67	69	71	73	75	78
3	81	83	85	88	90	92	94	96	99	101
4	103	105	107	110	112	114	116	119	121	123
5	125	127	129	132	134	136	138	140	143	145
6	147	149	151	154	156	158	160	162	165	167
7	169	171	173	176	178	180	182	184	187	189
8	191	193	195	198	200	202	204	206	208	210
9	212	214	216	218	221	223	225	227	229	232
10	234	236	238	241	243	245	247	250	252	254
11	256	258	260	263	265	267	269	272	274	276
12	278	280	282	285	287	289	291	293	296	298
13	300	302	304	306	308	310	312	314	316	319
14	321	323	325	328	330	332	334	336	339	341
15	343	345	347	350	352	354	356	358	360	362
16	364	366	368	370	373	375	377	379	382	384
17	386	388	390	393	395	397	399	401	404	406
18	408	410	412	415	417	419	421	423	425	427
19	430									

Precautions. The proportions of the various solutions prescribed above must be preserved unchanged. The directions for heating and cooling must be followed meticulously because variations of time and temperature affect the results significantly.

SOMOGYI'S MICRO-MODIFICATION OF THE SHAFFER AND HARTMANN METHOD (38)

Somogyi has recommended a modification of the Shaffer and Hartmann method that permits the analysis of as little as 0.2 cc. of blood. The procedure is carried out on a 1/15 tungstic acid blood filtrate.

TABLE 51-B

For Shaffer-Hartmann-Somogyi Blood Sugar Method with Reagent No. 2

Amounts of glucose corresponding to titration values when 5 cc. of 1:10 blood filtrate and 5 cc. of copper reagent No. 2 are heated in water bath for fifteen minutes.

CUBIC CENTIMETERS OF 0.005 N THIOSULFATE B—A	TENTHS OF 1 CC. OF 0.005 N THIOSULFATE									
	0	1	2	3	4	5	6	7	8	9
	Milligrams of glucose in 100 cc. of blood									
0		7.5	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.0
1	29.5	32.0	34.5	37.0	39.0	41.5	44.0	46.0	48.5	50.5
2	53.0	55.0	57.5	59.5	62.0	64.0	66.5	69.0	71.0	73.0
3	75.5	78.0	80.0	82.0	84.5	87.0	89.0	91.5	93.5	96.0
4	98.0	100.0	102.5	105.0	107.0	109.0	111.5	113.5	115.5	118.0
5	120.0	122.5	125.0	127.0	129.0	131.5	134.0	136.0	138.0	140.5
6	143.0	145.0	147.0	149.0	151.0	154.0	156.0	158.0	160.0	163.0
7	165.0	167.0	169.0	171.0	174.0	176.0	178.0	180.0	182.0	185.0
8	187.0	189.0	191.0	194.0	196.0	198.0	200.0	202.0	204.0	207.0
9	209.0	211.0	213.0	215.0	218.0	220.0	222.0	224.0	226.0	229.0
10	231.0	233.0	235.0	237.0	240.0	242.0	244.0	246.0	248.0	251.0
11	253.0	255.0	257.0	260.0	263.0	265.0	267.0	269.0	271.0	274.0
12	276.0	278.0	280.0	283.0	285.0	287.0	290.0	292.0	294.0	296.0
13	298.0	300.0	303.0	305.0	307.0	309.0	312.0	314.0	316.0	318.0
14	320.0	322.0	325.0	327.0	329.0	331.0	334.0	336.0	338.0	340.0
15	342.0	344.0	347.0	349.0	351.0	353.0	355.0	358.0	360.0	363.0
16	364.0	366.0	369.0	371.0	373.0	375.0	377.0	380.0	382.0	384.0
17	386.0	388.0	390.0	392.0	394.0	396.0	398.0	400.0		

Reagents

An 0.0667 (1/15) N solution of sulfuric acid.

A 2.5 per cent solution of sodium tungstate, prepared by dilution from the usual 10 per cent Folin-Wu solution (see p. 65).

Copper reagent. Either one of the copper reagents described in the macro-procedure above may be used equally well. Calculation values for Reagent 1 can be found in table 52. There are no such factors available for Reagent 2.

A 1 N sulfuric acid solution.

A 0.005 N sodium thiosulfate solution standardized as described in the macro-procedure, above.

Procedure

Preparation of protein-free blood filtrate. Into a conical centrifuge tube measure 2 cc. of 0.0667 N sulfuric acid. Into this deliver 0.2 cc. of blood from a pipette, calibrated ***to contain*** this amount. Rinse the pipette by drawing the acid mixture to the mark and expelling it a few times, and then withdraw the pipette, while blowing through it, along the side of the tube. Mix the contents of the tube well by stirring with a *footed glass rod and, after a few moments, add exactly 0.8 cc. of 2.5* per cent sodium tungstate. Mix well by stirring, cover the tube with a cork, tin foil or rubber cap, and then centrifuge.

TABLE 52

FOR MICRO SHAFFER-HARTMANN-SOMOGYI BLOOD SUGAR METHOD WITH REAGENT 1

Amounts of glucose corresponding to titration values when 2 cc. of 1:15 blood filtrate and 2 cc. of copper reagent are heated in water bath for fifteen minutes.

CUBIC CENTIMETERS OF 0.005 N THIOSULFATE $B-A$	TENTHS OF 1 CC. OF 0.005 N THIOSULFATE									
	0	1	2	3	4	5	6	7	8	9
	Milligrams of glucose in 100 cc. of blood									
0		27	39	50	58	67	75	83	92	100
1	107	115	124	132	141	149	157	166	174	182
2	190	198	207	215	224	231	239	248	256	264
3	272	280	289	297	305	313	321	330	338	346
4	354	362	370	379	387	395	403	412	420	428
5	436	444	452	460	469	477	485	493	502	510
6	518	527	534	542	551	558	566	575	582	590
7	599	607	615	623	632	639	647	655	663	671

Determination. Transfer 2 cc. of the filtrate to a clean test tube, 16 by 150 mm. in size, add exactly 2 cc. of copper reagent, mix by gentle shaking, cover the tube with a glass bulb and heat it in boiling water for ten minutes. The tube should be supported in a metal rack in the bath to prevent agitation of its contents. At the end of the ten minutes, cool the solution rapidly by immersion in cold water, not by shaking under running water. Add 2 cc. of 1 N sulfuric acid, shake the tube until the cuprous oxide is completely dissolved and, after a minute or two, titrate the solution with 0.005 N thiosulfate from a micro-burette, as described above.

For the transfer of blood filtrate, a 2 cc. pipette is used, over the end of which a tuft of absorbent cotton has been twisted, to act as a filter.

Calculation

A blank determination is made on the reagents. The calculation is made as described for the macro form of this method above with the values in table 52, if copper Reagent 1 has been used.

An empirical curve of glucose equivalents may be constructed and used, as described under the macro-procedure, with either copper reagent. The aliquot used for analysis is equivalent to 0.133 cc. of blood.

Precautions. Any agitation of the test tubes from the beginning of heating in the water bath up to the addition of the acid before the titration should be avoided to minimize reoxidation of cuprous oxide by air. The precautions which have been mentioned under the macro-procedure must be carefully observed.

TITRATION FERRICYANIDE METHOD OF HAGEDORN AND JENSEN (22)

The method depends on the quantitative reduction of potassium ferricyanide by sugar in an alkaline solution, and the iodometric titration of the excess ferric salt.

$$2\ K_3\,Fe(CN)_6 + 2\ KI = 2\ K_4Fe(CN)_6 + I_2$$

Reagents

0.1 N sodium hydroxide, prepared weekly by dilution from 2 N sodium hydroxide.

0.45 per cent zinc sulfate, prepared weekly by dilution from 45 per cent zinc sulfate solution.

0.005 N alkaline ferricyanide. 1.65 grams of potassium ferricyanide and 10.6 grams of fused sodium carbonate are dissolved in water and made up to 1 liter. This solution will keep in a dark bottle for two months. The potassium ferricyanide must be purified. This can be done by the method of Folin described above, or in the following manner. The crude crystals are first washed with cold water and then dissolved in hot water. The solution is filtered through a small filter paper which has previously been washed with hot water. The filtrate is allowed to crystallize in a procelain dish surrounded by ice water. The crystals are freed from excess water by blotting with washed filter paper and are then dried in an oven at 50°C. The salt should not be exposed to strong light, but kept in a dark bottle. It should not contain ferric salts or ferrocyanide.

Test for ferric salts. To 5 cc. of a 5 per cent solution of ferricyanide add 1 drop of 10 per cent sulfuric acid and 1 drop of 1 per cent potassium ferrocyanide. 0.01 mg. Fe in the form of Fe^{+++} ions gives a blue color.

Test for ferrocyanide. To 1 cc. of ferricyanide add a few drops of 1 per cent fresh ferric chloride and a few drops of 1 N HCl. 0.02 mg. of ferrocyanide will give a blue color.

The sodium carbonate is recrystallized and fused in a platinum crucible.

Folin and Malmros (18) suggest that the carbonate and ferricyanide be made up as separate solutions, because the alkali hastens decomposition of ferricyanide.

Iodide and zinc reagent. Five grams of potassium iodide, 10 grams of zinc sulphate and 50 grams of sodium chloride are dissolved in 200 cc. of water. This solution is not stable. Stock solution is made up without the KI and the potassium iodide is added to portions as needed. When free iodine is discernible in this solution most of it can be removed by filtration through a thick filter paper. Small amounts of iodine are taken care of in the blank analysis. This solution must not contain iron.

Acetic acid. Three cubic centimeters of glacial acetic acid made up to 100 cc. with water. The acid must be free from iron.

Starch solution. One gram of soluble starch dissolved in 100 cc. of saturated sodium chloride solution with the aid of heat (see p. 34).

0.005 N sodium thiosulfate. 0.7 gram are dissolved in water and made up to 500 cc. This solution is allowed to stand for several days before using. It is standardized frequently with potassium iodate solution (see p. 33).

0.005 N standard potassium iodate solution. Ten cubic centimeters of standard 0.1 N iodate (p. 32) are diluted to 2 liters.

Procedure

Precipitation of blood proteins. 0.1 cc. of blood is delivered from a capillary pipette calibrated ***to contain*** this amount, into a test tube containing 1 cc. of 0.1 N NaOH and 5 cc. of 0.45 per cent zinc sulfate solution. The pipette is rinsed twice with the solution in the test tube. The test tube is then placed in a boiling water bath for four minutes, cooled and the contents filtered through washed cotton on a 3 to 4 cm. wide funnel into a short, wide, test tube (30 by 90 mm.). The residue is washed 3 times with 3 cc. portions of water. The filtrate can be set aside for as long as one hundred hours at room temperature at this time without affecting results. Folin and Malmros (18) have called attention to the fact that the size of the cotton filter makes some difference. They

claim that only about 20 mg. of cotton should be used and must be only loosely inserted in the funnel. If larger amounts are used some sugar will be lost.

Reduction of ferricyanide by blood filtration. To the protein-free filtrate (about 14 cc.) exactly 2 cc. of the alkaline ferricyanide solution are added. The tube is then immersed in boiling water for exactly fifteen minutes. Usually a series of tubes is heated at once; it is convenient to use a cylindrical copper rack, such as is employed by bacteriologists, to hold the tubes. The solutions are cooled by immersing the tubes in cold water for three minutes. After cooling there is no danger of reoxidation of ferrocyanide even if the tubes are allowed to stand for several hours exposed to the air at room temperature.

Blank determination. A blank determination is performed on all reagents without the addition of blood filtrate. This determination is carried through in all details exactly like the analysis of the blood filtrate, including the filtering through cotton.

Titration. To the previously heated ferricyanide-sugar solution are added 3 cc. of the iodide-zinc solution and 2 cc. of acetic acid. The quantity of these need not be measured very exactly, and the solution can be set aside for a short time if desired. The solution is titrated by adding thiosulfate from a Bang micro burette (see p. 13) until the yellow color has almost disappeared. One drop of starch solution is then added and thiosulfate run into the solution drop by drop until the red-blue color just vanishes. The number of cubic centimeters of thiosulfate used are then read on the burette, A, if a blood filtrate is analyzed; B, if the determination is a blank without sugar.

Standardization of thiosulfate solution. A solution of 3 cc. of the iodide-zinc solution, 2 cc. of acetic acid, 15 cc. of water, and exactly 2 cc. of the standard potassium iodate solution is used. Thiosulfate is added from the micro burette until most of the iodine has disappeared. Then one drop of starch is added and the solution is carefully titrated until the red-blue color vanishes. The factor for the 0.005 N thiosulfate is $\frac{2}{\text{cc. thiosulfate}}$.

Precautions. The potassium ferricyanide must be added quantitatively from a calibrated Ostwald pipette. The titration with sodium thiosulfate must be carefully performed, using a calibrated micro burette divided in intervals of 0.02 cc.

Calculation

The amount of glucose present in the sample may be calculated from the equation

$$\mathrm{Gl} = 0.1735\,C + \frac{0.005\,C}{2.27 - C}$$

TABLE 53

FOR HAGEDORN-JENSEN BLOOD SUGAR TITRATION

The table gives milligrams of glucose per 100 cc. of blood indicated by A, the cc. of 0.005 N thiosulfate used, when the blank, B, is exactly the theoretical 2.00 cc. If, because of reducing impurities in the reagents, the blank is less than 2.00 cc., the difference, $2.00 - B$, is subtracted from A as a correction, and the corrected A is applied to the table.

A CUBIC CENTIMETERS OF 0.005 N THIOSULFATE	HUNDREDTHS OF 1 CC. OF 0.005 N THIOSULFATE									
	0	1	2	3	4	5	6	7	8	9
	Milligrams of glucose in 100 cc. of blood									
0.0	385	382	379	376	373	370	367	364	361	358
0.1	355	352	350	348	345	343	341	338	336	333
0.2	331	329	327	325	323	321	318	316	314	312
0.3	310	308	306	304	302	300	298	296	294	292
0.4	290	288	286	284	282	280	278	276	274	272
0.5	270	268	266	264	262	260	259	257	255	253
0.6	251	249	247	245	243	241	240	238	236	234
0.7	232	230	228	226	224	222	221	219	217	215
0.8	213	211	209	208	206	204	202	200	199	197
0.9	195	193	191	190	188	186	184	182	181	179
1.0	177	175	173	172	170	168	166	164	163	161
1.1	159	157	155	154	152	150	148	146	145	143
1.2	141	139	138	136	134	132	131	129	127	125
1.3	124	122	120	119	117	115	113	111	110	108
1.4	106	104	102	101	99	97	95	93	92	90
1.5	88	86	84	83	81	79	77	75	74	72
1.6	70	68	66	65	63	61	59	57	56	54
1.7	52	50	48	47	45	43	41	39	38	36
1.8	34	32	31	29	27	25	24	22	20	19
1.9	17	15	14	12	10	8	7	5	3	2

where Gl = milligram of glucose in the sample analyzed and C = cc. of 0.005 N thiosulfate equivalent to the ferricyanide reduced by sugar.

$$B - A$$

as in the Shaffer-Hartmann-Somogyi calculation. B represents the cubic centimeters of thiosulfate required to titrate a blank analysis, and A cubic centimeters required in titration of the blood filtrate.

The number of milligrams of sugar per 100 cc. of blood may be read directly from table 53.

GASOMETRIC FERRICYANIDE METHOD OF VAN SLYKE AND HAWKINS (42)

This method is described in the chapter on gasometric methods, page 408.

TIMING FERRICYANIDE METHOD OF HAWKINS AND VAN SLYKE (24, 25)

This method uses the time required for the decolorization of a colored oxidizing agent as a measure of the sugar content of a solution. This principle was first utilized by Cole (12) for the analysis of urine, a modification of Fehling's solution being boiled with the urine until the blue color of the copper disappeared. Hawkins and Van Slyke (25) afterwards applied the same principle for use with the yellow ferricyanide reagent which they had developed for gasometric sugar determinations. Hawkins (24) subsequently refined the method to such a degree that duplicate analyses can be performed on the filtrate from 0.2 cc. of blood. The method is extremely simple and rapid, permitting the analysis of several blood filtrates in five minutes without other apparatus than a procelain casserole and some small test tubes. The accuracy of the method, ± 5 per cent of the sugar present, is sufficient for ordinary purposes.

Reagents

Potassium ferricyanide solution. 0.5 gram of potassium ferricyanide, 75 grams of anhydrous potassium carbonate and 75 grams of potassium bicarbonate are dissolved in water and diluted to 1 liter. The solution is filtered, even if it appears clear. In dark colored, glass-stoppered bottles it usually keeps for several months, but it should either be renewed or tested with known glucose solutions every 2 months. The potassium ferricyanide must be weighed accurately, the carbonates only roughly.

The ferricyanide should contain no ferrocyanide. It may be tested as follows. To 10 cc. of 0.5 per cent potassium ferricyanide, add 0.5 cc. of a 0.5 per cent ferric chloride solution and 1 drop of 1 N hydrochloric acid. If as much as 0.1 per cent of the ferricyanide has become reduced to ferrocyanide a green color will appear when the ferric chloride is added. High grade commercial products should give no reaction to this test. Ferricyanide may be purified, if necessary, by the method of recrystallization described for Folin's micro colorimetric technique above (see p. 463).

Tungstic acid solution (mixed reagents of Folin and Wu). One volume of 10 per cent sodium tungstate and 1 volume of the $\frac{2}{3}$ N sulfuric acid used in

the Folin-Wu precipitation of proteins (see p. 66) are mixed with 8 volumes of distilled water. This solution is not permanent, but can be used for two weeks in spite of a slight precipitate which appears after a somewhat shorter time.

Procedure

Precipitation of blood proteins. When samples of capillary blood are to be analyzed, a sufficient number of small rubber-stoppered tubes are prepared each containing exactly 2 cc. of the tungstic acid precipitating reagent described above. The blood drops forming on the incised finger or ear lobe are drawn by capillary attraction into a 0.200-cc. capillary pipette (calibrated *to contain* this amount), which is at once emptied into one of the test-tubes. The pipette is rinsed twice by drawing the tungstic acid up into it. The test-tube is then stoppered and shaken. The blood is diluted 1:11 by this procedure. After two minutes the mixture is filtered through a dry filter paper (4.5 cm.) into a test-tube (15 by 100 mm.).

If the blood is known to be hyperglycemic, a portion of the filtrate is diluted with an equal volume of water.

When other blood analyses are to be made which require a Folin-Wu filtrate where the blood is diluted 1:10, this filtrate can be used instead of the 1:11 blood filtrate.

Decolorization of ferricyanide by blood filtrate. 0.5 cc. of filtrate is pipetted into a thin walled Pyrex test-tube, of 9 by 90 mm. outside measure[7] followed by 0.5 cc. of ferricyanide solution. Both solutions must be measured accurately. The ferricyanide should be added last, in order to facilitate its mixture with the lighter filtrate. The tube is shaken to mix the two solutions and is then placed in water already gently boiling in a white glazed casserole. The casserole has a diameter of 95 mm. so that the tubes rest slanting with their mouths on the edge, but cannot slip into the water. Two or three tubes may be heated at once. A tube containing distilled water is immersed with the other tubes to facilitate by comparison the detection of the moment when the ferricyanide solutions are decolorized. The time in seconds for each tube is taken, preferably with a stop-watch, from the moment the tube is immersed in the boiling water until the last trace of yellow disappears.

[7] The tubes are made from standard thin walled Pyrex glass tubing with an internal diameter of 8 mm. The thickness of the glass wall is 0.6 mm. Thin walls are important. The tubes may be obtained from Eimer and Amend, New York.

With the prescribed amount of ferricyanide and size of test-tubes, 50 mg. of sugar per 100 cc. of blood decolorize the reagent in 520 seconds, and 300 mg. of glucose per 100 cc. of blood will decolorize it in 76 seconds, when the blood filtrate represents a 1:11 dilution of the blood. If blood filtrate represents a 1:10 dilution for blood, the decoloration times are correspondingly shorter, as shown by figure 63. It is possible to work within the above time limits. However, if the reagent is decolorized in less than 110 seconds (equivalent to more than 150 mg. per cent of blood sugar for blood diluted

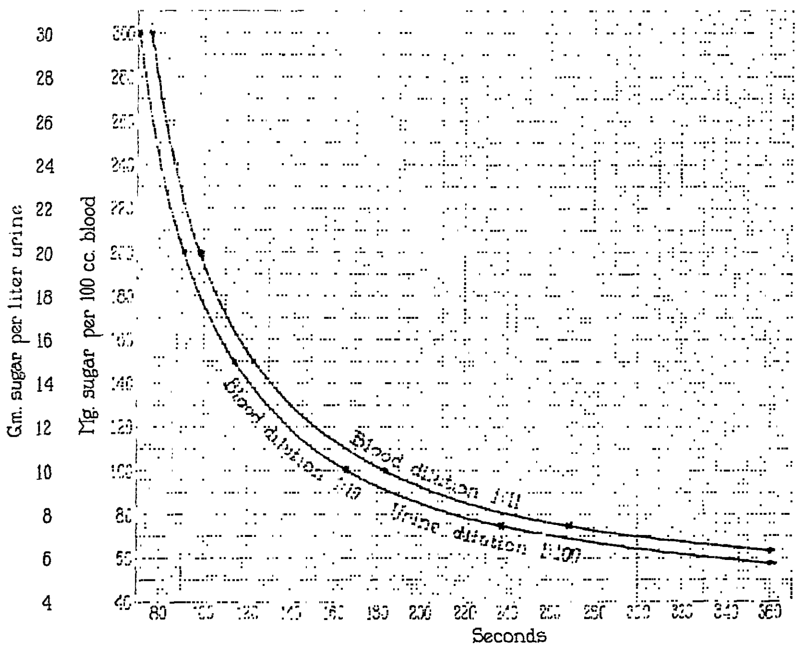


FIG. 63. Curves for graphic calculation of blood and urine sugar determined by Hawkins-Van Slyke time reduction method. Ordinates by use of the appropriate curve represent grams of sugar per liter urine when urine is diluted 1:100 or milligrams of sugar per 100 cc. of blood when blood is diluted 1:10 or 1:11. If other dilutions are used, sugar contents represented by the ordinates are multiplied or divided accordingly; e.g., if by reason of hyperglycemia blood is diluted twice as much as usual (1:20 or 1:22 instead of 1:10 or 1:11), multiply the sugar content read on the curve by 2.

1:11, and to more than 140 mg. per cent of blood sugar for blood diluted 1:10) it is best to dilute another portion of blood filtrate with an equal volume of water and to repeat the analysis with the diluted filtrate in order to obtain a longer decolorizing period and more exact results.

A little practice is necessary to train the eye in judging the moment at which the yellow color vanishes.

Calculation

The number of milligrams of sugar per 100 cc. of blood is read directly from the appropriate curves shown in figure 63 when the 0.5 cc. of filtrate used represents a 1:11 or a 1:10 dilution of the blood. When the filtrate is twice as dilute (hyperglycemic blood) the blood sugar content indicated by the appropriate curve is doubled.

It is desirable that the analyst check the curves of figure 63 by analysis of standard glucose solutions. The size and thickness of the test tubes has some influence on the time required for reduction, and the tubes available may not be exact duplicates of those used by Hawkins. If the deviation of results from the curves of figure 63 is significant, the analyst prepares his own curves.

DETERMINATION OF FERMENTABLE SUGAR IN BLOOD BY MEASURING THE DECREASE IN REDUCING SUBSTANCES CAUSED BY YEAST FERMENTATION

The principle underlying this procedure is the determination of reducing material in two samples of the same specimen of blood, one of which has been subjected to yeast fermentation under conditions which insure rapid and complete removal of glucose. The difference between the two determinations represents fermentable sugar which, there is reason to believe, is almost entirely glucose.

Fermentation has, in the past, yielded conflicting results which have been discussed by Hiller, Linder and Van Slyke (28). The chief source of the irregularities appears to have been the use of prolonged fermentation periods, twelve to twenty-four hours. If yeast is permitted to grow so long in blood it may also yield to the blood reducing products of its own manufacture. The possibility of bacterial action further complicates the reactions. To avoid such errors Hiller, Linder and Van Slyke (28) introduced the principle of using sufficiently large quantities of yeast to insure complete fermentation in a short time, thirty minutes or less. Under these conditions comparatively consistent results have been obtained by Folin and Svedberg (19), Somogyi (39), Benedict (7, 9) and Raymond (34). Somogyi (39) improved the technique by using suspensions of yeast cells which had been freed from adherent reducing substances by repeating washing.

Folin and Svedberg (19) showed that the fermentation could be carried out in Folin-Wu blood filtrates as well as in whole blood. The application to

filtrates Somogyi (39) believes to be preferred, because he has found that blood from certain subjects with pathological conditions reacts with yeast to produce reducing substances. However, the filtrates from such bloods ferment with yeast in a normal manner.

Somogyi (39) and Van Slyke and Hawkins (43) have shown that yeast cells are quite impermeable to the non-fermentable reducing substances that are found in blood filtrates. Also the volume of water which adheres to the washed packed cells is negligible. Consequently, in the calculation of non-fermentable reducing material in properly prepared yeast-blood filtrates no allowance need be made for the volume occupied by the yeast cells, which, except for their ability to absorb fermentable sugar, may be regarded as inert, impermeable, solid particles.

Reagents

Sodium tungstate and sulfuric acid used in the Folin-Wu precipitation of proteins, see page 65.

Washed yeast cells. A portion of Fleischmann's yeast is pulverized and suspended in about 4 times its weight of water. The cells are thrown down in centrifuge tubes and are then washed by resuspending and centrifuging with 4 or 5 successive portions of water. The washed cells are finally suspended in a weight of water 4 times as great as that of the portion of crude yeast taken at the beginning.

Procedure

Precipitation of proteins. From a sample of freshly drawn blood, preferably as much as 5 cc., if material permits, the proteins are removed by the usual Folin-Wu tungstic acid precipitation (see p. 65). The filtrate is divided into two portions.

Determination of reducing substances. One of these portions is analyzed for sugar directly by any one of the methods described above to obtain ***total reducing substances.*** The second is analyzed by the same method after it has been subjected to yeast fermentation in the following manner.

Removal of fermentable sugar. Of the suspension of washed yeast cells a volume, approximately equal to that of the blood filtrate to be fermented, is centrifuged. The supernatant liquid is decanted and adherent water is removed from the walls of the tube and from the surface of the packed yeast with a roll of filter paper. The sample of blood filtrate is introduced into the tube and mixed with the yeast. After the mixture has stood at room temperature for fifteen minutes it

is centrifuged. Portions of the supernatant fluid are analyzed for ***non-fermentable reducing substances*** by the same method which was used for the determination of total reducing substances.

Control determination of reducing substances in yeast. A control on the yeast is made by suspending a similar portion of centrifuged yeast in water instead of blood filtrate for fifteen minutes and determining reducing substances in the supernatant water.

Van Slyke and Hawkins (43) found that yeast should be extracted with water rather than tungstic acid for the control analysis. In the tungstic acid precipitation of blood, proteins and tungstic acid mutually precipitate one another, leaving a filtrate which contains little tungstate and is only slightly acid. Such filtrates extract from washed yeast the same amount of reducing material that water does. Tungstic acid solution which has not been rendered comparatively non-acid by protein precipitation, on the other hand, extracts from yeast considerably greater amounts of reducing material.

Calculation

Indicating total reducing substances as T, non-fermentable substances found after yeast treatment as N, and the reducing substances extracted from yeast in the blank analysis as Y:

$$\text{Fermentable blood sugar} = T - (N - Y).$$

DETERMINATION OF FERMENTABLE BLOOD SUGAR BY THE AUTOGLYCOLYSIS METHOD. HILLER, LINDER AND VAN SLYKE (28); VAN SLYKE AND HAWKINS (43)

Hiller, Linder and Van Slyke (28) and Folin and Svedberg (19) have found that spontaneous glycolysis at 38° for twenty hours or more and short fermentation by yeast removed from blood the same amount of reducing substances.

Procedure

A. One sample of fresh blood is analyzed at once for total reducing material, T, by one of the blood sugar methods described above.

B. Another sample of the same whole blood, in a stoppered tube, is incubated at 38° for twenty to twenty-four hours. At the end of this time it is analyzed for non-fermentable reducing material N by the same method which was applied to **A.**

Calculation

$$\text{Fermentable sugar} = T - N$$

PARTIAL REMOVAL OF NON-GLUCOSE REDUCING MATERIAL BY THE ZINC PRECIPITATION METHOD OF SOMOGYI (40)

Somogyi has found that by precipitation of blood proteins by zinc salts filtrates can be obtained which are almost entirely free from reducing substances other than glucose (non-fermentable reducing material). These non-glucose materials are precipitated with the proteins. Similar results have been secured by West, Scharles and Peterson (44) with mercury salts; but their procedure are more complicated than that of Somogyi. (For further discussion of the Somogyi precipitation method see "Precipitation of Proteins," p. 69).

Since Somogyi's work appeared in 1930 Benedict has published (9a) a method (described above on page 456) which when applied to the usual tungstic acid blood filtrates indicates no more non-fermentable material (equivalent to 4 to 8 mg. glucose per 100 cc. blood) than was shown by former methods with the Somogyi filtrate. There is consequently less need for a special technique, such as Somogyi's, for preparing blood filtrates for analysis in hypoglycemic conditions, than there was at the time of Somogyi's paper.

Reagents

Solution I. Ten per cent zinc sulfate. One hundred grams of $ZnSO_4 \cdot 7\,H_2O$ to 1 liter.

Solution II. 0.5 N sodium hydroxide.

The two solutions must be so related that when 10 cc. of the zinc sulfate are titrated with the sodium hydroxide, 10.8 to 11.2 cc. of the alkali are required to produce a permanent pink color with phenolphthalein. The 10 cc. of Solution I are diluted with 50 to 70 cc. of water before the titration; the alkali is added slowly with continuous shaking.

Procedure

For the preparation of 1:10 ***blood*** filtrates, lake 1 volume of blood with 7 volumes of water.. Add 1 volume of the zinc sulfate, mix, then add 1 volume of the 0.5 N alkali.

To prepare 1:10 filtrates of ***corpuscles,*** lake 1 volume of corpuscles with 5 volumes of water. Precipitate with 2 volumes of the zinc sulfate followed by 2 volumes of the alkali.

To precipitate the proteins of blood ***plasma*** or ***serum*** at 1:10 dilution, introduce 1 volume of plasma or serum into 8 volumes of water, and use 0.5 volume of each precipitant.

BIBLIOGRAPHY

1. Bang, I.: Zur Methodik der Zuckerbestimmung. II. *Biochem. Z.*, 1913, **49**, 1.
2. Bang, I.: Ein Verfahren zur Mikrobestimmung von Blutbestandteilen. *Biochem. Z.*, 1913, 49, 19.
3. Bang, I.: Der Blutzucker, Wiesbaden, 1913. (J. F. Bergmann).
4. Benedict, S. R.: The detection and estimation of reducing sugars. *J. Biol. Chem.*, 1907, **3**, 101.
5. Benedict, S. R.: The detection and estimation of glucose in urine. *J. Am. Med. Assoc.*, 1911, **57**, 1193.

5a. Benedict, S. R.: A reagent for the detection of reducing sugars. *J. Biol. Chem.*, 1909, **5**, 485.

6. Benedict, S. R.: The determination of blood sugar. *J. Biol. Chem.*, 1925, **64**, 207.
7. Benedict, S. R.: The estimation of sugar in blood and normal urine. *J. Biol. Chem.*, 1926, **68**, 759.
8. Benedict, S. R.: Determination of sugar in blood. *J. Biol. Chem.*, 1929, **83**, 165.
9. Benedict, S. R.: The determination of blood sugar. II. *J. Biol. Chem.*, 1928, **76**, 457.

9a Benedict, S. R.: The analysis of whole blood. II. The determination of sugar and of saccharoids (non-fermentable, carbohydrate-reducing substances). *J. Biol. Chem.*, 1931, **92**, 141.

10. Benedict, S. R., and Osterberg, E.: A method for the determination of sugar in normal urine. *J. Biol. Chem.*, 1918, 34, 195.
11. Bertrand, G.: Le dosage des sucres réducteurs. *Bull. Soc. Chim. Paris*, 1906, **35**, 1285.
12. Cole, S. W.: Practical physiological chemistry, Cambridge, 1920.
13. Everett, M. R.: Determination of sugar in blood. I. Observations upon Benedict's alkaline copper solution. *J. Biol. Chem.*, 1929, **82**, 369.
14. Fehling, H.: Die quantitative Bestimmung von Zucker and Stärkmehl mittlest Kupfervitriol. *Annalen der Chemie.*, 1849, 72, 106.
15. Folin, O.: A new blood sugar method. *J. Biol. Chem.*, 1928, **77**, 421.
16. Folin, O.: Supplementary note on the new ferricyanide method for blood sugar. *J. Biol. Chem.*, 1929, **81**, 231.

16a. Folin, O., and Berglund, H.: A colorimetric method for the determination of sugars in normal human urine. *J. Biol. Chem.*, 1922, **51**, 209.

17. Folin, O., and Malmros, H.: An improved form of Folin's micro method for blood sugar determinations. *J. Biol. Chem.*, 1929, **83**, 115.
18. Folin, O., and Malmros, H.: Blood sugar and fermentable sugar as determined by different methods. *J. Biol. Chem.*, 1929, **83**, 121.
19. Folin, O., and Svedberg, A.: The sugar in urine and in blood. *J. Biol. Chem.*, 1926, **70**, 405.
20. Folin, O., and Wu, H.: (a) A system of blood analysis. *J. Biol. Chem.*, 1919, **38**, 106. (b) Supplement I. A simplified and improved method for the determination of sugar. *J. Biol. Chem.*, 1920, **41**, 367.
21. Friedenson, M.; Rosenbaum, M. K.; Thalheimer, E. J., and Peters, J. P.: Cutaneous and venous blood sugar curves. I. In normal individuals after insulin and in liver disease. *J. Biol. Chem.*, 1928, **80**, 269.
22. Hagedorn, H. C., and Jensen, B. N.: Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. *Biochem. Z.*, 1923, **135**, 46.

23. HAWKINS, J. A.: Reducing powers of different sugars for the ferricyanide reagent used in the gasometric sugar method. *J. B. C.*, 1929, **84**, 79.
24. HAWKINS, J. A.: A micro time method for determination of reducing sugars, and its application to analysis of blood and urine. *J. Biol. Chem.*, 1929, **84**, 69.
25. HAWKINS, J. A., AND VAN SLYKE, D. D.: A time method for determination of reducing sugars, and its application to analysis of blood and urine. *J. Biol. Chem.*, 1929, **81**, 459.
26. HERBERT, FRIEDA K.; BOURNE, MARGHERITA C., AND GROEN, JUDA: The effect of glutathione on the determination of blood sugar. *Biochem. J.*, 1930, **24**, 291.
27. HILLER, A.: A quantitative test for small amounts of sugar in the urine. *J. Biol. Chem.*, 1917, **30**, 125.
28. HILLER, A.; LINDER, G. C., AND VAN SLYKE, D. D.: The reducing substances of the blood. *J. Biol. Chem.*, 1925, **64**, 625.
29. KIEFER, E. D.: A bedside method for determination of blood sugar. *J. Am. Med. Ass.*, 1925, **84**, 589.
30. LEWIS, R. C., AND BENEDICT, S. R.: A method for the estimation of sugar in small quantities of blood. *Proc. Soc. Exp. Biol. Med.*, 1913, **11**, 57; *J. Biol. Chem.*, 1915, **20**, 61.
31. MYERS, V. C., AND BAILEY, C. V.: The Lewis and Benedict method for the estimation of blood sugar, with some observations obtained in disease. *J. Biol. Chem.*, 1916, **24**, 147.
32. NEUBERG, C.: Kleinere Mitteilungen verschiedenen Inhalts. III. Über einige Reaktionen vergorener Zuckerlösungen. *Biochem. Z.*, 1910, **24**, 430.
33. NEUBERG, C., AND HILDESHEIMER, A.: Über zuckerfreie Hefegärungen. I. *Biochem. Z.*, 1911, **31**, 170.
NEUBERG, C., AND TIR, L.: Über zuckerfreie Hefegärungen. II. *Biochem. Z.*, 1911, **32**, 323.
NEUBERG, C., AND KARCZAG, L.: Über zuckerfreie Hefegärungen. III *Biochem. Z.*, 1911, **36**, 60.
NEUBERG, C., AND KARCZAG, L.: Über zuckerfreie Hefegärungen. IV (Carboxylase, ein neues Enzym der Hefe) *Biochem. Z.*, 1911, **36**, 68.
34. RAYMOND, A. L., AND BLANCO, J. G.: Blood sugar determination and separation of sugars with live yeast. *J. Biol. Chem.*, 1928, **79**, 649.
35. ROTHBERG, V. E., AND EVANS, F. A.: Studies with the Folin and Wu blood sugar determination. *J. Biol. Chem.*, 1923, **58**, 435.
A modified Folin and Wu blood sugar method. *J. Biol. Chem.*, 1923, **58**, 443.
36. SHAFFER, P. A., AND HARTMANN, A. F.: The iodometric determination of copper and its use in sugar analysis. I. Equilibria in the reaction between copper sulfate and potassium iodide. *J. Biol. Chem.*, 1921, **45**, 349.
37. SHAFFER, P. A., AND HARTMANN, A. F.: II. Methods for the determination of reducing sugars in blood, urine, milk and other solutions. *J. Biol. Chem.*, 1921, **45**, 365.
38. SOMOGYI, M.: Notes on sugar determination. *J. Biol. Chem.*, 1926, **70**, 599.
39. SOMOGYI, M.: Reducing non-sugars and true sugar in human blood. *J. Biol. Chem.*, 1927, **75**, 33.
40. SOMOGYI, M.: A method for the preparation of blood filtrates for the determination of sugar. *J. Biol. Chem.*, 1930, **86**, 655.

41. SUMNER, J. B.: Dinitrosalicylic acid: A reagent for the estimation of sugar in normal and diabetic urine. *J. Biol. Chem.*, 1921, **47**, 5.
42. VAN SLYKE, D. D., AND HAWKINS, J. A.: A gasometric method for determination of reducing sugars and its application to analysis of blood and urine. *J. Biol. Chem.*, 1926, **79**, 739.
43. VAN SLYKE, D. D., AND HAWKINS, J. A.: Gasometric determination of fermentable sugar in blood and urine. *J. Biol. Chem.*, 1929, **83**, 51.
44. WEST, E. S.: Scharles, F. H., AND PETERSON, V. L.: The determination of true sugar in blood. *J. Biol. Chem.*, 1299, **82**, 137.

CHAPTER IX

Lipoids

GENERAL PRINCIPLES

Methods for the determination of lipoids have certain common procedures. In all, the lipoids are first extracted by some lipoid solvent from the material to be analyzed; from the extract the various lipoids or lipoid groups are then separated and estimated. In no analyses is it more important to test the methods by control determinations on known substances.

Extraction

Extraction of the lipoids can be effected by a variety of methods and solvents. Of the latter, extensive investigations by Kumagawa and Suto (22) have shown that hot alcohol is probably the most efficient. Its value lies chiefly in the fact that it is miscible with water. Although alcohol extracts the lipoids more rapidly and completely than other solvents do, it also extracts many other substances. To separate the lipoids from these it is necessary to remove the alcohol and to reextract the residue with some more specific solvent. For this purpose ether or petroleum ether is generally employed.

Dry extraction. In all earlier methods the material to be analyzed was first dried completely and powdered. The powder was then extracted either by direct admixture with the lipoid solvent and subsequent filtration or by reflux distillation by some modification of the Soxhlet procedure.

For the anlysis of liquids, like blood, admixture with some inert material facilitates drying and permits more readily the penetration of the solvent by insuring the exposure of a larger surface to its action. In Bang's (2) method for the determination of blood lipoids, Harnes' (20) phosphatide method, and Leiboff's (23) and Ling's (25) cholesterol methods the blood is absorbed and dried on filter paper before extraction. Myers and Wardell employ plaster of Paris for the same purpose. Some observers have, however, experienced difficulty in preventing caking of the plaster and in transferring it quantitatively to the extraction vessel. For this reason Leiboff (23), Ling (25) and others have preferred Bang's filter paper method.

Wet extraction. From liquid or semi-liquid media it is possible to extract lipoids without drying by merely shaking the material with proper lipoid solvents. The method of wet extraction was proposed by Roese and Gottlieb (33) for the analysis of blood and milk. . It has since been employed in

various modifications by Meigs and Marsh (28) for milk; by Saxon (35), Holt, Courtney and Fales (21) and Fowweather (17) for feces; and by Bloor (5, 9, 12) and Stewart and White (37) for blood.

The method involves extraction with two lipoid solvents. One, alcohol, is miscible with water and serves both as a protein precipitant and to render the lipoids soluble. The other, ether, has a greater solvent power for fatty substances and is only slightly miscible with water. When the alcoholic aqueous solution is shaken with the ether, the lipoids for the most part pass into the ether, in which they are more soluble. The ether layers out on standing and can be separated with its contained lipoids. The latter can be purified subsequently by resolution in other solvents.

Although tradition has given preference to the more conventional dry extraction methods, there is evidence that wet extraction is actually not only easier, but also more efficient and accurate. In the analysis of stools Fowweather (17) found that lipoids were more completely recovered by wet extraction. Moreover, he showed that in the process of drying some neutral fat was hydrolyzed and became saponified.

Saponification. Kumagawa and Suto (22), proposed saponification of fatty acids in the material as a preliminary to extraction. The alkali used for saponification of the fats also causes hydrolysis of other organic material. After saponification the fatty acids are liberated by acidification. This procedure renders the lipoids readily extractable by ethyl ether. The ether extract is purified by reextraction with petroleum ether. From the latter the fatty acids may be separated from non-saponifiable material by shaking the petroleum ether extract with potassium ethylate and water. The soaps pass into the alcoholic water solution, while the non-saponifiable material is left in the petroleum ether. This method and its various modifications and adaptations appear to give excellent results for total fatty acids, but does not permit fractionation of the lipoids.

Determination of total lipoids

Total lipoids were first estimated, after extraction or purification by one of the methods outlined above, by evaporating the organic solvent from a known aliquot of the extract and weighing the dried residue. This is the principle which has been employed by Roese and Gottlieb (33), Meigs and Marsh (28), Saxon (35) and others.

In place of weighing the extracted lipoids Bloor (5) in 1914 proposed nephelometric determination. The precipitate produced by pouring an ether-alcohol solution of lipoids into water was compared with that produced by similar treatment of a known amount of fat. By this method it

was possible to determine the fats in small samples of blood. The principle is, however, open to serious objections, which were pointed out especially by Csonka (14) and Blix (4). The clouding produced by precipitates of lipoids is dependent not only on the quantity but also on the quality of fatty substances present in the mixture. Nephelometric estimation is, therefore, accurate only when the proportions of different lipoids in the solutions to be compared are constant, a condition only roughly approximated in blood.

Bang (2), in 1918, introduced a new principle for determination of the extracted lipoids, which has since been adopted with certain improvements by Bloor (9). In this method the extracted lipoids are oxidized by dichromate in the presence of concentrated sulfuric acid. The excess dichromate is then titrated iodimetrically.

Backlin has recently introduced a gasometric micro procedure. He oxidizes extracted lipoids with sulfuric acid and silver chromate till all the carbon is converted into CO_2, and then determines the CO_2 gasometrically. The method is described on page 433 among gasometric analyses. Backlin claims that it gives theoretical yields of CO_2 and is less liable to error than the Bang-Bloor chromate titration method.

Determination of total fatty acids

Fatty acids occur in the lipoids of blood, tissues and feces, not only in combination with glycerine as neutral fats, but also in phosphatides and in cholesterol esters. In blood and living tissues free fatty acids and soaps probably do not exist; but in feces they are produced from neutral fat and fatty acid esters by the action of lipase and bacteria. By treatment with alkali, fats, lipoids, and fatty acid esters can be saponified; that is, their fatty acids are released from organic combination and converted into alkali salts. From these fatty acid salts the fatty acids can be released by acidification. Although the soaps are soluble in, or at least miscible with, water, the fatty acids themselves are not. The latter can, therefore, be extracted by organic solvents from the water suspensions obtained when the soap solutions are acidified, and can be estimated by titration with sodium alcoholate. By this means the total fatty acid content of materials can be determined. Such procedures have been applied by Stewart and White (37) and Stoddard and Drury (38) to the determination of the total fatty acid content of blood. Of these titration methods Stewart and White's suffers the disadvantage that the measure of the fatty acids is a relatively small difference between large amounts of alkali and acid. This disadvantage seems to have been successfully avoided by Stoddard and Drury.

Determination of free fatty acids, soaps, and neutral fats in extracts

In the analysis of stools, where fatty acids appear not only as neutral fats, but also free and as soaps, the estimation of the relative proportions of the three forms may give information of some importance, because it aids in the evaluation of the efficiency of the various factors involved in the digestion of fat. The procedure by which fatty acids are fractionated is illustrated by Fowweather's (17) modification of Saxon's (35) wet extraction method for analysis of feces. In this method two samples of stool, to one of which acid is first added, are treated with alcohol and then extracted with ethyl ether. The ether extracts are then dried, reextracted with petroleum ether, and filtered. The dried residues are then weighed. The weight of the acidified extract gives a value for total lipoids, while from that of the neutral extract the weight of free fatty acid and neutral fat is obtained. The difference between the two represents fatty acids present as soaps. The extract obtained without acidification is then redissolved, and the free fatty acids are determined by titration with sodium alcoholate. The difference between the weight of the whole neutral extract and the weight of its free fatty acids estimated from the titration gives the weight of neutral fat.

Similar procedures are employed for fractionation of the fatty acids in the original method of Saxon (35) and in those of Holt, Courtney and Fales (21) and others.

Determination of phosphatides

Most of the older methods for the determination of phosphatides are based on the same general principles and involve three separate procedures: first, the extraction or separation of the lipoids; second, the destructive digestion of these lipoids and the liberation of phosphoric acid; and finally the analysis of the residue for phosphoric acid. The methods employed for the extraction of the lipoids are those used for fat extractions in general; the digestion methods are similar to those commonly used in the determination of total phosphoric acid in biologic material; the analysis of the final residue may be carried out by any suitable phosphate method. In Bloor's most recent method the phospholipoids are separated by precipitation with magnesium chloride and acetone from the solution of total lipoids, and the lipoid content of the precipitate is measured by the chromate oxidation and titration method instead of by phosphorus determination. Doubtless this oxidation could be combined also with Backlin's gasometric determination of the CO_2 formed in place of Bloor's titration of the excess chromate.

Determination of cholesterol

Windaus (43) in 1909 showed that free cholesterol reacts quantitatively with digitonin in alcoholic solution to form an insoluble compound of stable composition, while cholesterol esters are not precipitated. On the basis of this reaction he developed, for the determination of total cholesterol and its free and combined fractions, a gravimetric technique. This required such large quantities of blood and was so time consuming that it was little employed for clinical studies. The principle of digitonin precipitation, however, still remains the basis of all methods that aim to differentiate free and combined cholesterol. *The digitonin method determines free cholesterol but not cholesterol esters.*

In 1910 Grigaut (19) presented a colorimetric method which utilized the Liebermann-Burchard (24) reaction, the development of a green color when cholesterol or its esters are treated with acetic anhydride in the presence of sulfuric acid. This procedure was simplified and made more suitable for general clinical use by Autenrieth and Funk (1) who are often given credit for its origination. *This colorimetric method determines total cholesterol, both free and esterified.*

Weston (41), in 1912, proposed a colorimetric technique in which the Salkowski (34) color reaction was employed. With the exception of Schmidt (36), however, investigators have preferred the Liebermann-Burchard principle.

In general, all methods involve two processes: first, the isolation of the cholesterol by extraction, and, second, the analysis of the extract. Autenrieth and Funk (1) saponified the blood or plasma with strong potassium hydroxide before extracting it with chloroform. The process of digestion and extraction was time consuming and complicated, and the final chloroform solutions showed at times a tendency to assume a yellowish tint which could not be eliminated. In 1915 Bloor (6) described a simplified adaption of the Autenrieth and Funk method which could be employed in conjunction with his system for the analysis of the lipoid constituents of the blood. In the following year (7) he introduced further modifications, especially the elimination of preliminary saponification. These techniques gave higher values than that of Autenrieth and Funk or than digitonin precipitation, partly because of the presence of other color producing substances (26, 29, 42) and partly because of the propensity of the blood extracts to take on yellowish and brownish tints. This Mueller (29) found could be diminished or prevented in most specimens by washing the chloroform extracts with water and then dehydrating with anhydrous sodium sulfate. In 1922 Bloor, Pelkan and Allen (12) altered the cholesterol procedure, reintroducing pre-

liminary saponification. This, however, Bloor (9) has again abandoned in his most recent publication. Even if Bloor's methods do yield somewhat excessive values for cholesterol at times, their adaptability to his system for the quantitation and fractionation of all the blood lipoids gives them a peculiar value.

For the determination of cholesterol alone in blood or plasma Myers and Wardell (30) presented a colorimetric method which appears to give values that agree satisfactorily with those obtained by digitonin precipitation, an agreement that, at present, must be considered the best criterion of accuracy. The blood or plasma, mixed with plaster of Paris, is extracted directly with chloroform by distillation with a reflex condenser. Baumann and Holly (3) and MacAdam and Shiskin (27) have endorsed the method. Leiboff (23) and Ling (25) have found it difficult to keep the plaster of Paris mixture finely divided and also to transfer it quantitatively to the extraction apparatus. They prefer to absorb the blood on fat-free filter paper and have described convenient apparatus for the extraction of the latter by chloroform.

Szent-Györgi has proposed both a gravimetric (39) and a titrimetric (40) procedure. The former is a micro-adaptation of the Windaus method; the latter depends upon the oxidation of digitonin-cholesteride by an excess of potassium chromate and subsequent iodimetric titration of the residual chromate. Okey (32) from Bloor's laboratory has recently proposed a modification of this chromate method. Doubtless Backlin's gasometric oxidation method (see p. 433) could also be applied to micro-determination of the digitonin-cholesteride.

Bloor and Knudson (11), Gardner and Fox (18) Okey (32), and Turner (40a) have presented methods for the separate determination of cholesterol and cholesterol esters. In these procedures the free cholesterol is separated by digitonin precipitation.

Lipoids in Feces

SOAPS, FAT, AND FREE FATTY ACIDS OBTAINED BY WET EXTRACTION. FOWWEATHER'S (17) MODIFICATION OF SAXON'S METHOD (35)

This procedure is for application to the stools as voided, without preliminary drying.

Special reagents required

Redistilled ethyl ether. This should leave no residue after evaporation to dryness.

Redistilled 95 per cent alcohol.

Petroleum ether, boiling below 60°, redistilled, and leaving no residue when evaporated to dryness. This can best be prepared by fractional distillation of commercial petroleum ether with a Clarke's (13) column. Reject fractions that distill above 60°. Wash the distillate that distils below 60° with concentrated sulfuric acid and redistil it with an ordinary condenser; 50-cc. portions, evaporated to dryness, should leave no residue.

Benzene (C_6H_6).

0.5 per cent alcoholic solution of phenolphthalein.

0.1 N sodium ethylate. This is prepared by dissolving in 1 liter of redistilled absolute alcohol 2.3 grams of freshly cut, bright, metallic sodium, The solution may be standardized by titration against 0.1 N acid, with alcoholic phenolphthalein as indicator. It is preferable, however, to standardize with stearic or oleic acid dissolved in benzene, by the method described below for the titration of fatty acids. The solution should be kept cool in the dark and should be discarded when it becomes much colored.

Preparation of stools

The total stool specimen is thoroughly mixed, with the aid of a mortar and pestle, if necessary. If the feces are liquid, about 5 grams of the mixed sample are poured into each of three weighed, stoppered weighing bottles. After the bottles have been weighed again one of them is placed on a steam bath or in a drying oven (temperature 95° to 100°) and the contents are dried to constant weight. This gives the per cent of dry matter in the stools. Each of the two remaining fractions is washed into a 100 cc. glass-stoppered cylinder of uniform bore. To one are added 3 cc. of concentrated hydrochloric acid and water enough to make a total volume of 30 cc.; to the other water alone to make the same volume.

If the stool is solid, after thorough mixing about 10 grams are transferred to a porcelain evaporating dish containing a glass rod 2 to 3 inches in length, flattened at one end. Dish and rod are weighed together in advance and are reweighed with the sample of feces. With the aid of the glass rod 2 to 3 grams of stool are transferred to each of two 100-cc. glass-stoppered cylinders of uniform bore. The dish with the rod and the remainder of the stool are reweighed after each transfer. Care must be taken not to contaminate the necks of the cylinders with feces when these are introduced. The evaporating dish, rod and remainder of the stool are then dried to constant weight on a steam bath or in a drying oven. The samples in the cylinders are then treated with acid and water as described above.

Extraction and weighing of total fat and of free fatty acids plus neutral fat

To each cylinder add 20 cc. of ethyl ether. Shake vigorously for five minutes. After they have stood a few moments introduce 20 cc. of alcohol into the neutral cylinder and 17 cc. into the acid cylinder. Mix the alcohol with the other contents of the cylinders by a sharp rotary motion. Cool the cylinders to room temperature in running water. Shake the contents vigorously for five minutes and then allow them to stand until the ether layer separates. Separation may be

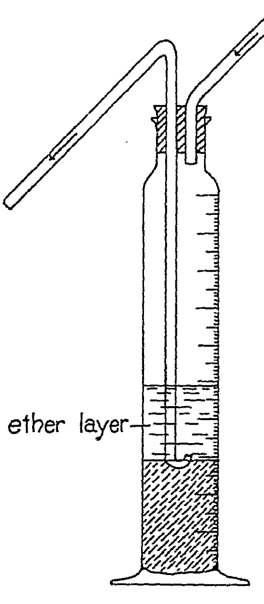


FIG. 64. Separating cylinder for removal of ether in stool extraction (Meigs and Marsh (28)).

expedited by occasional rotation of the cylinder or by adding a few drops of alcohol or ether.

When separation is complete the ether layer is blown off as completely as possible into a fat extraction flask by means of the apparatus illustrated in figure 64. The type of extraction flask used is that in figure 66. Another 20 cc. of ether is then added, the cylinder is shaken for five minutes and the ether allowed to separate as before. This is then blown off and the washing process is repeated. The sides of the flask are then washed down with three successive 5-cc. portions of ether.

The combined extract and washings in the extraction flask are now evaporated to dryness. The dry residue is then dissolved in petroleum ether and filtered through fat free filter paper into a weighing bottle. Flask and filter paper are washed with additional petroleum ether.

The petroleum ether is now evaporated or distilled off and the residue is dried to constant weight and weighed. The residue from the extract of the acidified stool mixture represents ***total fat,*** and the residue from the extract of the unacidified stool represents ***free fatty acids + neutral fat.***

Titration of free fatty acids

The extract of the unacidified stool is dissolved in about 50 cc. of benzene and warmed almost to the boiling point. It is then titrated, while still hot, with 0.1 N sodium alcoholate, with 2 to 3 drops of phenolphthalein as indicator. The titration should be continued until the color of the indicator no longer deepens. The amount of indicator used must be the same as that employed in the standardization of the alcoholate. The color of the indicator fades rapidly as the solution cools, even after the true end point has been reached.

Blank determinations must be made in which the whole procedure, including extractions, is carried out with all reagents, the fatty material alone being omitted.

Calculation of results

One cubic centimeter of 0.1 N sodium alcoholate titrates 28.2 mg. of oleic acid or 28.4 grams of stearic. Hence

(Cubic centimeters 0.1 N sodium alcoholate) $\times$ 28.3 = milligrams of free fatty acids.

A. The weight of total fat.
B. The weight of free fatty acids + neutral fat.
C. The weight of free fatty acids (calculated from titration).
$D = A - B$ = fatty acids present as soaps.
$E = B - C$ = neutral fat.

SOAPS, FAT, AND FREE FATTY ACIDS OBTAINED BY WET EXTRACTION. HOLT, COURTNEY, AND FALES' MODIFICATION OF SAXON'S (35) METHOD

This procedure is for application to stools which have been prepared for analysis by preliminary drying and pulverizing, as described on page 78. The material is mixed with water again before the fats are extracted.

Special reagents required

Dilute hydrochloric acid (1 part of concentrated hydrochloric acid to 3 parts of water). The other reagents are the same as those used in the wet extraction procedure described above.

Preparation of stools

The stools are thoroughly dried and pulverized by the usual method (see p. 78). One-gram samples of the dried powder are transferred to two small, weighed dishes and weighed. To one of these is added 10 cc. of the hydrochloric acid, to the other an equal amount of water.

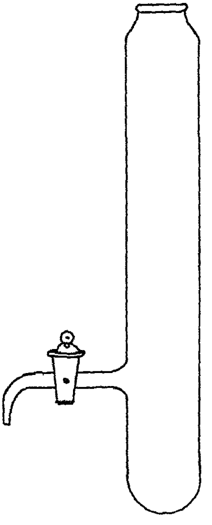


FIG. 65. Röhrig tube for the separation of ether in the extraction of lipoids from stools, milk, or blood.

The dishes are then placed on a water bath or steam bath until their contents are thoroughly heated and disintegrated. Their contents are then transferred to Röhrig tubes (see figure 65) or to glass stoppered cylinders like those used in the Fowweather method. Enough water is added to make a total volume of 30 cc. or to fill the Röhrig tube half way to the pet-cock. Twenty cc. of alcohol or enough to bring the upper fluid level almost to the pet-cock of the Röhrig tube is then introduced.

Extraction

Twenty-five cubic centimeters of ethyl ether are added and the cylinder or tube is shaken vigorously for half a minute. Twenty-five cubic centimeters of petroleum ether are introduced and the mixture is shaken for another half minute. It is then allowed to stand until the ether layer is separated and clear, when the layer of ethers is drawn off or blown off. The extraction is repeated once with 20 cc. of both ethers added separately as before, and a second time with 30 cc. of mixed ether (recovered from previous washings).

From the combined extracts the ether is distilled off with precautions to ***avoid ignition of ether vapors*** that may escape.

The residue is then dried over night on a water bath and finally in an oven at 95° to 100° for several hours, until it attains constant weight.

The free fatty acids are titrated in the neutral extract in the manner described under the wet extraction method. The methods of calculation in the two procedures are identical.

Lipoids in Blood, Serum, and Plasma

PREPARATION OF ALCOHOL-ETHER EXTRACT OF TOTAL LIPOIDS BY THE METHOD OF BLOOR (5, 6, 9, 12)

Reagents required. Alcohol-ether mixture: 3 parts of 95 per cent alcohol and 1 part of ether. Both must be redistilled.

Procedure

Into 40 cc. of alcohol-ether mixture in a 50-cc. volumetric flask, run in a slow stream, while rotating the flask, 3 cc. of plasma or serum. If larger amounts of extract are required take 5 cc. of plasma and 75 cc. of alcohol-ether in a 100-cc. flask. The precipitate of protein thus produced should be homogeneous and finely flocculent.

Immerse the flask in boiling water, rotating it continuously (to prevent superheating and bumping) for a few seconds after its contents have begun to boil. Cool in running water to room temperature. Make the volume up to 50 cc. (or 100 cc.) with alcohol-ether mixture and mix by shaking. Filter the mixture through fat-free filter paper. To obtain the greatest possible quantity of filtrate fold the filter paper containing the precipitate on the funnel and press out the filtrate with a glass rod. Aliquots of this filtrate are used for the determination of the various lipoid fractions.

Whole blood. If whole blood is to be analyzed a larger proportion

of alcohol-ether must be used. Three cubic centimeters of blood are run into 75 cc. of alcohol-ether in a 100-cc. volumetric flask, boiled, and made up to 100 cc.

DETERMINATION OF TOTAL LIPOID FATTY ACIDS AND CHOLESTEROL BY CHROMATE OXIDATION (BLOOR (9))

Special reagents required

0.1 N sodium thiosulfate (prepared in the usual manner, see p. 32).

1.0 N (1/6 molar) potassium dichromate. Dissolve 49.03 grams of pure potassium dichromate in water and dilute to 1000 cc. This solution may be standardized against the thiosulfate by the method of titration outlined below for the determination of fat.

Ten per cent potassium iodide solution.

One per cent starch solution prepared in the usual manner (see p. 34).

Silver dichromate in concentrated sulfuric acid, prepared according to the method of Nicloux (31). Dissolve 5 grams of silver nitrate in 25 cc. of water in a 100-cc. centrifuge tube. Add 5 grams of potassium dichromate dissolved in about 50 cc. of water. Centrifuge the mixture. Wash the precipitate twice with water to remove nitrate. Dissolve the cake of precipitate, without drying, in 500 cc. of concentrated sulfuric acid.

Petroleum ether (prepared as described under Fowweather's method for fecal lipoids).

Sodium ethylate, approximately 1.0 N. Dissolve 2.3 grams of freshly cut, bright metallic sodium in 100 cc. of absolute alcohol, keeping the solution cool during the process. The reagent should be kept cool in the dark and should be discarded when it becomes much discolored.

Dilute sulfuric acid. 1 Volume of concentrated sulfuric acid to 3 volumes of water.

Saponification

Fifteen to 20 cc. (enough to contain about 5 mg. of total lipoid) of the alcohol-ether lipoid solution, prepared as described above on page 495, are measured into a 100-cc. Erlenmeyer flask, and 2 cc. of sodium ethylate are added. The mixture is evaporated on a water bath until the odor of alcohol disappears. The last traces of alcohol vapor should be swept out of the flask by a gentle current of air.

Extraction of fatty acids and cholesterol

To the pasty residue add 1 cc. of dilute sulfuric acid. Heat the acid mixture on a water or steam bath for one minute. Into the hot mixture pour 10 cc. of petroleum ether which is thereby made to boil. Rotate

the flask gently at boiling temperature on the water bath for two or three minutes. At the end of this time pour the solvent as completely as possible from the watery residue into a 25-cc. volumetric flask. The heating and extraction are repeated with 5-cc. portions of petroleum ether, the sides of the flask being washed down and the ether poured off completely each time, until the volumetric flask is almost full. The flask is then cooled to room temperature, filled to the mark with petroleum ether, the stopper is inserted tightly and the contents are mixed. After this treatment both aqeuous and ether solutions should be clear.*

Oxidation

Ten cubic centimeters aliquots of the petroleum ether solution are measured into 125-cc. glass-stoppered flasks. The solvent is evaporated completely, the last traces being blown out of the cylinder with a gentle current of air. Five cubic centimeters of silver chromate reagent, accurately measured, are added, followed by exactly 3 cc. of normal potassium dichromate. The flask must be rotated during the introduction of these reagents.

A blank determination with all reagents except the fatty material must be run through at the same time and under the same conditions as the plasma analysis.

After its contents have been well mixed by rotation, the flask, loosely stoppered, is set in an electric oven at $124° \pm 2°$ and left five minutes. It is then removed, stirred again by rotation, the stopper is tightly inserted, and the flask is replaced in the oven for ten to fifteen minutes. The mixture should remain definitely brown throughout the process of heating. If it becomes green or even distinctly greenish when the reagents are mixed or at the end of the first five minutes of heating it contains too little dichromate. At this point the addition of a second amount of silver and dichromate and a repetition of the heating for the usual period generally saves the determination; but a duplicate of a smaller aliquot should be run through as a check.

The temperature of the oven in which the oxidation is carried out must not vary more than 2° from 124° and this temperature must be maintained uniformly in all the flasks. The introduction into the oven of an iron plate on which all the flasks may stand aids in the maintenance of even heat. A flask like those in which the oxidation is carried out, containing a similar fluid and equipped with a standardized thermometer, should be placed in the oven with the other flasks as a check on the temperature control. In every determination, therefore, four flasks,

* No flame must be burning near the place where the extraction is carried out.

two containing duplicate samples of the material to be analyzed, one for the blank on reagents, and one holding the thermometer, should be placed in the oven at the same time.

If an oven is not available the flasks may be set on a steam bath which will give a temperature of 90° in them. In this case oxidation must be continued for a total time of 60 minutes.

When the period of heating is completed, the flasks are removed from the oven and the contents, while still hot, are diluted with 75 cc. of water.

Attaining complete oxidation under conditions such that a quantitative measurement is possible depends largely on adjustment of time and temperature of heating. The chromic acid if heated too high gives off its oxygen spontaneously to the air. The temperature must be so adjusted that this decomposition is negligible during the time of heating employed and yet that the reagent is in so sensitive a condition that the last traces of material are completely oxidized. The temperature of greatest sensitivity with least decomposition is in the neighborhood of 124° and both temperature and time of heating are fairly critical. Not more than 2° above or below 124° is allowable, although five minutes longer time than that specified makes only a negligible difference. After oxidation is finished the mixture is cooled, and is then diluted with 75 cc. of water.

Titration of excess dichromate

Ten cubic centimeters of 10 per cent potassium iodide are added, without stirring, which might cause loss of liberated iodine, and 0.1 N thiosulfate is gradually run in from a burette. During the titration the flask is at first rotated very gently. As the iodine content diminishes, however, it is rotated more forcefully. During the titration the color of the mixture changes from a muddy greenish brown to a light blue. The silver causes a precipitate; but this does not interfere with the reading of the end point. When the end point is nearly reached add a few drops of starch and continue in the usual manner, until the starch is decolorized.

Calculation

Bloor calculated theoretically that 1 mg. portions of material should be oxidized by the following amounts of 0.1 N dichromate, which he also found to be approximated experimentally: 1 mg. palmitic acid, 3.57 cc. of 0.1 N dichromate; 1 mg. oleic acid, 3.61 cc. of 0.1 N dichromate; 1 mg. cholesterol, 3.92 cc. of 0.1 N dichromate. From these values a factor for approximate calculation of fatty acids + cholesterol, may be calculated according to Bloor from the fact that cholesterol as found in plasma is ordinarily about

one-half the total fatty acids. Of such a mixture 1 mg. requires 3.7 cc. of 0.1 N dichromate.

The cubic centimeters of 0.1 N dichromate used for oxidation are found as the difference between B, the cubic centimeters of 0.1 N thiosulfate required to titrate the blank, and A, the cubic centimeters required for the unknown. Hence

$$\text{Milligrams of fatty acids + cholesterol in sample} = \frac{B - A}{3.7}.$$

A theoretically more exact calculation is made by determining, as described below, the cholesterol content of the extract oxidized, and subtracting its 0.1 N dichromate equivalent, C, from the cubic centimeters of dichromate oxidized by fatty acids + cholesterol. The difference, representing dichromate oxidized by fatty acids alone, is divided by 3.6. The value of C is obtained by multiplying by 3.92 the number of milligrams of cholesterol in the amount of lipoid taken for oxidation.

$$\text{Milligrams of fatty acids in sample} = \frac{B - A - C}{3.6}.$$

Precautions

The alcohol-ether and the petroleum ether must be completely driven off after the saponification and the extraction respectively, because both solvents are oxidized by dichromate. However, the air current used to remove the last traces of these substances must not be too forceful nor must it be applied too long for fear of oxidizing some of the fatty acids. A current of CO_2 or N_2 would be preferable.

DETERMINATION OF TOTAL LIPOID FATTY ACIDS BY SAPONIFICATION AND TITRATION METHOD OF STODDARD AND DRURY (38)[1]

Special reagents required

Coarse sand, previously boiled with acid, washed, dried and extracted with ether.

Concentrated sodium hydroxide prepared by mixing equal weights of pure sodium hydroxide and water and permitting the solution to stand in a paraffin lined bottle until the carbonate has settled to the bottom.

30 volume per cent hydrochloric acid (30 cc. of concentrated acid diluted to 100 cc.)

10 per cent NaOH.

[1] We are indebted to Dr. Stoddard for reading and correcting the following description.

5 per cent sodium chloride solution (neutralized to methyl red).
0.02 N sodium hydroxide.
1 per cent alcoholic solution of phenolphthalein.
0.3 per cent thymol blue in 50 per cent alcohol

Extraction of lipoids

Extraction by alcohol-ether is carried out by the method of Bloor, 5 cc. of whole blood or plasma being made up to 100 cc. with the alcohol-ether.

Saponification

Evaporate 75 cc. of the alcohol-ether extract in a 100-cc. beaker, introducing only 50 cc. at first. Add a few grains of coarse sand to prevent bumping. Place the beaker on an electric stove at low heat, on the cover of a steam bath, or elsewhere, so that the ether will evaporate while the solution does not boil perceptibly. (***Caution.*** Evaporation should be done where there is a good draft to carry off ether vapors, and in the absence of flames.) When the ether has been driven off place the beaker in a gentle current of steam and continue evaporation until the volume of the alcoholic solution is reduced to about 30 cc. Add 0.1 cc. of concentrated sodium hydroxide, mix, add a few more grains of sand, cover with a watch glass, and allow the mixture to boil gently for twenty to thirty minutes to saponify the fatty acids. Remove the cover glass, drop in a small piece of litmus paper and acidify the solution with 30 per cent hydrochloric acid. Restore just to alkalinity with 10 per cent sodium hydroxide. It is desirable not to have an excess of alkali present during subsequent evaporation to dryness. Evaporate to dryness to get rid of all alcohol. Add 15 cc. of water, place the beaker on the steam bath and stir to dissolve the soaps. While the contents are still hot add a drop of thymol blue indicator followed by enough 30 per cent hydrochloric acid to give a faint pink color. Set the beaker for ten minutes in cold water. Then swirl the solution almost continuously for five minutes to cause better separation of the fatty acids.

Filtration of fatty acids

Filters should be prepared beforehand in the following manner. A Gooch crucible of the smallest size (top 28 mm., bottom 18 mm. in diameter) is set in a rubber washer which fits over the top of a small funnel. The stem of the funnel passes through a rubber stopper which

fits in a 500-cc. suction flask. A paper pulp emulsion is made by shaking a piece of soft filter paper, such as Schleicher and Schüll black ribbon, no. 589, in 300 to 400 cc. of distilled water. This emulsion is vigorously shaken and immediately poured into the crucible while strong suction is on. This is repeated until a filter layer about 1 mm. thick is produced. Tamp down this layer carefully with the end of a glass rod. Allow the larger masses of filter pulp fibers in the emulsion to settle out; then pour on successive amounts of the thin upper suspension of isolated shreds, keeping on strong suction and tamping occasionally, until the filter is sufficiently dense to offer definite resistance to the suction. Remove the crucible from its rubber washer, dry in an air oven at 110° for fifteen minutes and cool before using.

Set up a row of the funnels which fit the crucible washers. Place a crucible in its washer in a funnel. With a test-tube under the funnel pour into the crucible some of the fatty acid suspension. If the filtrate is not perfectly clear return it to the crucible and refilter it. If filtration does not begin in a few minutes transfer the crucible and funnel to a filter flask, with a test-tube under the funnel, and start gentle suction. After filtration has begun it is continued without suction. When the fatty acid suspension is filtered and the crucible drained, wash the precipitate with 4 cc. of 5 per cent sodium chloride, neutralized to methyl red. For this purpose use a pipette to run the salt solution down the walls of the beaker; then, tipping the beaker, rinse its sides more thoroughly with the aid of a bent glass rod. Pour this rinsing into the crucible, rinsing the sides of the latter by means of the rod. Wash repeatedly until the filtrate from one washing can be neutralized to phenolphthalein with not more than 0.05 cc. of 0.02 N sodium hydroxide. Three washings are usually required.

Resolution of the fatty acids

The crucible is placed again on the suction flask, in which is placed under the funnel a test tube of convenient height, calibrated at 1-cc. intervals from 10 to 15 cc. The walls of the beaker are washed down with 5 cc. of alcohol, which is then heated to boiling and poured into the crucible. Any fatty acid fragments on the walls of the crucible are quickly loosened with the glass rod. When the alcohol has nearly run out, moderate suction is applied. The beaker and crucible are washed out twice more with 3-cc. portions of alcohol, which are brought to a boil each time. Finally the outsides of the crucible and the funnel are washed in the same manner.

Titration

After the addition of a few grains of sand the filtrate is boiled for one minute. It is then cooled in a beaker, the volume of alcohol is noted, and 3 drops of 0.3 per cent thymol blue in 50 per cent alcohol are added. Titrate with 0.02 N sodium hydroxide to a pure blue color which remains practically unchanged, with no yellow tinge, while it is stirred for two minutes with a stopper in the mouth of the tube to avoid absorption of CO_2.

Ten cubic centimeters of alcohol are boiled down to the same volume reached by the boiled solution of fatty acids and are titrated as a blank.

Calculation

A represents the cubic centimeters of 0.02 N alkali used in the titration. B represents the cubic centimeters of 0.02 N alkali used to titrate the blank. C is an additional correction equivalent to 0.005 cc. of 0.02 N alkali, which must be subtracted from A to correct for the effect upon the end point of each cc. of water added with the alkali. Hence $A - C = 0.995\ A$. The calculation is:

$$\text{Millimoles fatty acid in sample titrated} = 0.02\ (0.995\ A - B)$$

To translate into milligrams of fatty acid, multiply millimols by 277, the average molecular weight of the fatty acids as they usually occur in blood.

When 5 cc. of blood are used and 75 cc. of filtrate evaporated, so that the sample titrated represents 3.75 cc. of blood, the calculation becomes:

$$\text{Millimoles of fatty acid per liter blood} = 5.30\ A - 5.33\ B.$$

$$\text{Milligrams of fatty acid per 100 cc. blood} = 147\ A - 148\ B.$$

DETERMINATION OF PHOSPHOLIPOIDS BY ESTIMATION OF PHOSPHORUS IN TOTAL LIPOIDS

Lipoid phosphorus may be determined in the Bloor alcohol-ether lipoid extract by methods described in the section on the determination of organic phosphorus in chapter 31.

DETERMINATION OF PHOSPHOLIPOIDS BY ISOLATION AND OXIDATION ACCORDING TO BLOOR (10)

The phospholipoids are precipitated from petroleum ether solution by adding acetone and magnesium chloride, the other lipoids remaining in solution. The lipoid in the precipitate is determined by oxidation with

silver dichromate, the excess of which is measured by titration with thiosulfate, as in Bloor's total lipoid method already described.

For plasma or serum the Bloor alcohol-ether lipoid extract may be used.

For whole blood extraction Bloor recommends the use of hot alcohol alone. Five cubic centimeters of blood are run with continuous stirring into about 75 cc. of redistilled 95 per cent alcohol in a 100-cc. volumetric flask. This is heated to boiling on a water bath and allowed to continue boiling gently, with occasional stirring, for five minutes. It is then cooled, made up to volume with alcohol and filtered through fat-free filter paper.

Special reagents required

Petroleum ether, prepared as described in Fowweather's method for fecal fat.

Redistilled acetone.

A saturated solution of magnesium chloride in redistilled alcohol.

Moist ether. Ether shaken with water until it is saturated. All ether must be peroxide-free. To test for peroxide shake the ether with half its volume of 10 per cent potassium iodide slightly acidified with sulfuric acid. If more than a faint yellow color develops the ether must be rejected or purified. Ether may be freed from peroxide by either of the following methods:

1. Distil the ether with a short fractioning column, retaining only the first two-thirds which distils over.

2. Wash the ether by shaking with 10 per cent potassium iodide acidified with sulfuric acid, adding thiosulfate to prevent accumulation of excess of free iodine. Continue shaking and decolorizing until no further color develops on the addition of potassium iodide. Wash the ether with water and distil.

The peroxide-free ether should be kept in the dark.

Silver dichromate reagent, potassium iodide, and *sodium thiosulfate* solutions are the same as those used for Bloor's oxidative determination of fatty acids described above.

Isolation of phospholipoids

A 20-cc. aliquot of the alcohol-ether extract of plasma or the alcoholic extract of blood is measured into a small beaker and evaporated to dryness. The residue is extracted by gentle boiling with at least three successive small portions of petroleum ether, which are, in turn, decanted into a 15-cc. graduated centrifuge tube until the total volume of liquid in the tube is 10 cc. The mixture is centrifuged and the liquid

transferred quantitatively to another centrifuge tube. It is then concentrated to 2 cc. by immersing the centrifuge tube in a beaker containing about 1 inch of hot water. (A boiling tube, made by fusing to the end of a 6-inch length of 2-mm. stirring rod about $\frac{1}{2}$ inch of melting-point tube, is inserted into the centrifuge tube to prevent explosive boiling). To the 2 cc. of concentrated mixture are added 7 cc. of redistilled acetone and 3 drops of cold saturated alcoholic magnesium chloride and the whole is well stirred with a small stirring rod or by bubbling air through it. The phospholipoids are precipitated.* Centrifuge at about 1500 r.p.m. for five minutes. The acetone solution is now decanted, the precipitate rinsed once with acetone, and the tube allowed to drain. The precipitate is dissolved in 5 cc. of peroxide-free moist ether, solution being aided by stirring with a small glass rod. The final traces of undissolved residue and the small drop of aqueous magnesium chloride solution are separated by centrifuging for 3 minutes at about 1500 r.p.m. The ether is quantitatively transferred to a digestion flask like those used in the oxidative determination of fat, either by decantation or by means of a rubber bulb and a tightly fitting 2 hole stopper used as is indicated in figure 64. The tube is rinsed twice with 1 cc. portions of ether. The solvent in the flask is then driven off by evaporation, the last traces being blown out of the flask by a gentle current of air.

Oxidation and titration

Oxidation and titration are carried out by the procedures described for the oxidative determination of fat. A blank determination must be carried through on all reagents.

Calculation

$$\frac{\text{cubic centimeters of thiosulfate used in blank} - \text{cubic centimeters of thiosulfate used for unknown}}{3.0} =$$

milligrams of lipide in sample.

COLORIMETRIC DETERMINATION OF TOTAL CHOLESTEROL IN BLOOR'S SAPONIFIED EXTRACT OF BLOOD LIPOIDS (12)

Special reagents required

Chloroform, free from water and other impurities.
Acetic anhydride.
Concentrated sulfuric acid.

* Some samples of commercial acetone fail to precipitate phosphatides. The acetone should be tested with lecithin or other known phosphatide.

Standard cholesterol solution in chloroform. It is best to prepare in chloroform a stock standard solution of cholesterol of which each cubic centimeter contains 2 mg. From this other standards are prepared by dilution, as required. As standard and unknown must not differ greatly, it is well to prepare more than one standard with each test. The strength of these standards will depend on the cholesterol content of the aliquot of blood or plasma used for the development of the color reaction. Each cubic centimeter of normal blood or plasma contains about 2 mg. of cholesterol. The standards most often required are those that contain, for analysis of plasma, 0.5, 1.0, and 2.0 mg. of cholesterol in 5 cc. portions of chloroform, and for whole blood 0.25, 0.5 and 1.0 mg. of cholesterol per 5 cc. of chloroform.

Meyers and Wardell (30) have suggested the substitution, for the cholesterol standard, of a standardized solution of naphthol green B.

Procedure

If of the lipoids only the cholesterol is to be determined the alcohol-ether extract representing about 0.3 cc. of blood or plasma is evaporated to dryness, and the dry residue is extracted by boiling with 3 successive portions of 1 cc. each of chloroform. The extracts are decanted into a 10-cc. glass stoppered graduated cylinder. The solution is cooled to room temperature, and is made up of 5 cc. by addition of more chloroform.

If the lipoids have, for the purpose of determination of fatty acids, already been saponified and redissolved in petroleum ether, as described above, 10 cc. of the petroleum ether extract are placed in a small Erlenmeyer flask and evaporated to dryness; the last traces of solvent are blown off with a gentle current of air. At least three successive small portions of chloroform are added, gently warmed to dissolve the residue, and decanted into a 10-cc. glass stoppered graduated cylinder. The solution is adjusted to room temperature and made up to the 5 cc. mark with chloroform.

To the 5 cc. of chloroform solution prepared in either way 1 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid are added. The cylinder is then stoppered and its contents are mixed by inversion. In a similar cylinder are placed 5 cc. of a standard solution of cholesterol in chloroform. To this also 1 cc. of acetic anhydride and 0.1 cc. of sulfuric acid are added, and the whole is mixed. The cylinders are then placed together either in the dark or in the same light conditions in which the colorimetric reading is to be made. After fifteen minutes they are compared in a colorimeter.

Total cholesterol, both free and present in the blood as ester, is determined in this, as in the other colorimetric methods described in this chapter.

Calculation

$$\text{Milligrams of cholesterol per 100 cc. blood} = \frac{100\ C}{V} \times \frac{S}{U}$$

C = milligrams of cholesterol in standard; V = cubic centimeters of blood represented by sample analyzed; S = reading of standard in the colorimeter; U = reading of unknown.

DETERMINATION OF TOTAL CHOLESTEROL IN SAPONIFIED LIPOID MIXTURE OF STODDARD AND DRURY (38)

The titrated mixture obtained at the end of the Stoddard and Drury fatty acid determination (p. 499) is transferred to a 100-cc. beaker and acidified with 3 N hydrochloric acid. After the addition of a few grains of sand it is evaporated to dryness on the steam bath. Ten cubic centimeters of chloroform are added; the residue is dissolved by stirring and allowed to stand 10 minutes, when it is filtered through a paper pulp Gooch crucible into a 20-cc. volumetric flask. The residue is extracted twice more with 5 cc. of chloroform for five minutes. The combined extracts are then made up to 20-cc. volume with chloroform and mixed. Two cubic centimeters are taken for a chloresterol determination, which is carried out according to the method of Bloor described above.

Although the fatty acids are present while the cholesterol is being determined, they give no color reaction nor do they interfere with the color reaction given by cholesterol.

Calculation is by the above formula.

DIRECT EXTRACTION AND COLORIMETRIC DETERMINATION OF TOTAL CHOLESTEROL. MYERS AND WARDELL (30) AND DE TONI (15)

If it is desired to determine only cholesterol small amounts of blood may be directly extracted with chloroform and colorimetric determination carried out in the usual manner.

Extraction

One cubic centimeter of blood or plasma is slowly run into a porcelain crucible or small beaker containing 4 or 5 grams of plaster of Paris. The mixture is stirred and dried, preferably in a drying oven. It is

then emptied completely into a small extraction shell 4 cm. long and this in turn is inserted into a short test tube 2.5 by 6 cm., with a number of holes in the bottom. This is attached to a large cork on a small reflux condenser, and tube and cork are inserted in the neck of a 150-cc. extraction flask containing 20 to 25 cc. of chloroform. Extraction is carried out for thirty minutes on an electric hot plate. The flask is then detached, the chloroform made up to a suitable volume (15 cc. is usually satisfactory) and filtered if necessary. The apparatus is illustrated in figure 66.

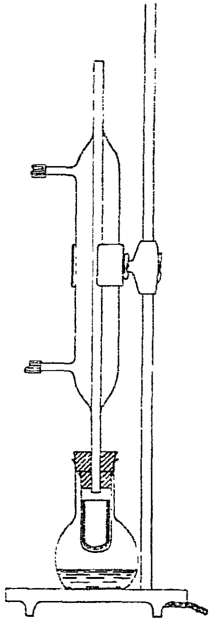


FIG. 66. Apparatus of Myers and Wardell (30) for the extraction of cholesterol from blood or serum.

Five cubic centimeters of the extract are then transferred to a dry test-tube or glass stoppered cylinder and treated in the usual manner described above. Myers and Wardell recommend the use of 2 cc. of acetic anhydride, instead of 1 cc.

De Toni (15) uses the same principle applied to the Folin-Wu tungstic acid precipitate of blood proteins. The precipitate, which contains all the lipoids as well as the proteins, is mixed with plaster of Paris in a

mortar, dried, pulverized, and extracted with chloroform as in the Myer-Wardell method. One portion of the extract can be used for cholestrol determination, another for determination of the phosphatides. De Toni's procedure makes for economy when both lipoids and substances in the Folin-Wu filtrate are to be determined.

DIRECT EXTRACTION AND COLORIMETRIC DETERMINATION OF TOTAL CHOLESTEROL. LEIBOFF (23) AND LING (25)

Because of difficulties they experienced in keeping the plaster of Paris finely divided and transferring it quantitatively in the method of Myers and Wardell, both Leiboff (23) and Ling (25) have proposed drying the blood on filter paper and have devised special apparatus to facilitate extraction.

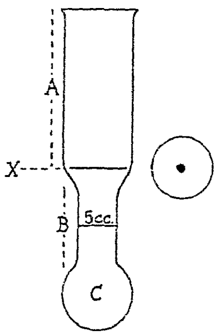


FIG. 67. Leiboff's (25) tube for the extraction of cholesterol from blood or serum in the procedure of Myers and Wardell. The entire length of the extraction tube is about 4 inches. The upper part *A* is 2 inches long and $\frac{7}{8}$ inches in diameter. The constricted part *B* is $\frac{1}{2}$ inch long in diameter and $1\frac{1}{4}$ inches long. The capacity of the bulb *C* is such as to hold a trifle less than 5 cc. of fluid, so that when exactly 5 cc. of fluid are placed in the tube, the meniscus of the fluid reaches about to the middle of the constricted portion *B* which is marked by a line. The filter paper disc X is prepared from Whatman fat-free extraction thimbles. A number of other filter papers were tried, but these were found to be the best for this purpose. The disc has a diameter of $\frac{3}{4}$-inch and a thickness of $\frac{1}{16}$-inch. It has a small opening in the center so that the vapors of chloroform may easily pass.

Leiboff has devised a tube in which both extraction of cholesterol and the development of the color reaction can be carried out without transfer. This is illustrated in figure 67. (Filter paper discs and tubes may be secured from the Empire Laboratory Supply Company, New York City.)

Five cubic centimeters of chloroform are placed in the extraction tube and the filter paper disc is inserted. 0.25 cc. of blood is then run

on to the disc with a pipette. The tube is attached to a reflux condenser and immersed, to a point above the level of the chloroform, in a beaker of hot or boiling water on an electric heater.

After thirty minutes' extraction it is detached from the condenser, the filter disc, *X*, is removed, the contents of the tube are cooled to room temperature in cold water, and enough chloroform is added to bring the volume of solution to the 5 cc. mark.

The actual determination of chloesterol is then carried out in the usual manner.

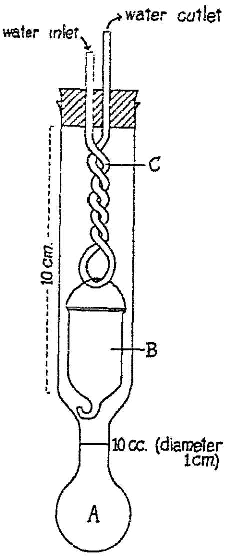


FIG. 68. Ling's (25) continuous extraction apparatus for use in the determination of cholesterol in blood and serum by the method of Myers and Wardell. Test tube (25 by 150 mm.) with a bulb, *A*, of about 9.5-cc. capacity at the lower end and a 10-cc. calibration mark on the neck of the bulb. *B*, a glass cup (20 by 30 mm.) used to hold the filter papers and suspended from the glass coil condenser by fine copper wire. *C*, a glass coil condenser inserted through a cork stopper.

Ling (25) has described a tube that combines extractor and condenser and permits development of color without transfer. This is illustrated in figure 68.

0.5 cc. of blood or plasma is run, by means of a pipette, on to two 7-cm. pieces of fat-free filter paper. These are then dried by suspending them on a clean copper wire over an electric hot plate. (Drying is not

absolutely essential). The filters are folded together so that they will drop into the glass cup, *B*. The cup is then hung on the condenser, 8 cc. of chloroform are placed in the tube, condenser and cup are attached, and the tube is inserted into boiling water which should come above the level of the chloroform. Extraction should be continued for forty minutes after chloroform begins to drip from the condenser. At the end of this time condenser and cup are detached, the tube is cooled under the tap, and enough chloroform is added to bring the volume of solution to 10 cc.

The determination is continued in the usual manner.

Precautions

The greatest difficulty in the colorimetric methods is the tendency for a yellow or brownish discoloration to appear from time to time in the chloroform extracts. This is probably due to the presence in the extracts of color-producing substances other than cholesterol, possibly, as Luden has suggested, bile derivatives. This difficulty is more frequently encountered when Bloor's alcohol ether extract is redissolved directly in chloroform. It is less common if chloroform extraction is preceded by saponification as in the Stoddard method, or is applied directly to dried blood or plasma, as in the method of Myers and Wardell and its modifications. Mueller (29) has shown that the discoloration can usually be eliminated if the chloroform extract is washed with water and then dehydrated with anhydrous sodium sulfate. Bloor (9) advises the use of a red glass filter in the colorimeter when a serious degree of discoloration occurs.

As in most other colorimetric procedures, accurate results can be obtained only if the colors of unknown and standard differ by not more than 30 per cent. Ling (25) says that if the colors differ greatly, correct readings can be obtained by diluting the stronger solution with chloform. It is, however, better practice to prepare more than one standard.

FREE CHOLESTEROL. ISOLATION AND OXIDATIVE TITRATION AS DIGITONIDE. OKEY (32) AND TURNER (40A)

Principle

Free cholesterol is precipitated as the digitonide, and is oxidized with dichromate by the method of Bloor, the excess dichromate being determined by iodometric titration. Okey washed the digitonide in a porous crucible, following the general principles of Windaus (43). Turner simplified the technique for micro analyses by substituting washing in centrifuge tubes.

Special apparatus

Fifteen-cubic centimeter centrifuge tubes with conical bottoms and with well fitted ground glass stoppers are used. They are easily prepared by grinding grass stoppers with abrasive into ordinary centrifuge tubes.

Reagents

Digitonin, a 1 per cent solution in alcohol.

Reagents for the chromate oxidation titration, listed on page 496, viz., standard thiosulfate and dichromate, KI and starch solutions, and silver dichromate in concentrated sulfuric acid.

Alcohol, 95 per cent.

Ether, redistilled.

Procedure

Precipitation of digitonid. An aliquot of Bloor's alcohol-ether extract (see p. 495) containing 0.5 to 1.5 mg. of free cholesterol is placed in the 15-cc. centrifuge tube. Precipitation is most favorable if the volume is 6 to 8 cc., and if the volume of extract taken exceeds this amount, it is reduced by placing the tube in a water bath at 70° until the necessary amount of evaporation has occurred. One cubic centimeter of the digitonin solution is added and the mixture is evaporated to dryness in an oven at 124° or in a water bath at 70°. Evaporation in a water bath is longer but safer. In an oven there is likelihood of the formation of an explosive mixture of ether vapor and air, particularly if the oven is small and the number of tubes considerable. If a water bath is used, the temperature must be kept down to 70° in order to avoid loss of material from bumping of the solution.

Washing the precipitate. The precipitate is washed, first with ether to remove fatty substances, then with water to remove excess digitonin and water-soluble impurities.

When the last traces of solvent have been evaporated, 10 cc. of redistilled ether are added at once, without removal of the digitonide particles from the sides of the tube. If the initial washing is accomplished without disturbing the precipitate, it becomes flakey and allows thorough subsequent washings. Two additional extractions are made with warm ether by mixing the digitonide and ether with a stirring rod. The solvent is removed each time by decantation after centrifugation at high speed to pack the solid material into the bottom of the tube. The last traces of ether are removed by warming the tube in a water bath.

The precipitate is then washed with warm water until the wash waters no longer present a foamy appearance. The water is decanted in the same manner as the ether, but the centrifugations must be carried on a longer time (thirty minutes) to insure packing of the precipitate, which has a greater tendency to remain in suspension in water than in ether. The residual moisture is finally removed by drying the washed precipitate in the oven.

Oxidation. Silver chromate reagent is added as described on page 496 for determination of total lipoids by dichromate oxidation. Intimate contact of the oxidative mixture with the digitonide is obtained by placing a short rod, of 2-mm. diameter, in the tube, and tapping the tube carefully. The stirring rod must be short enough to allow insertion of the ground stopper, and is not removed during the heating. The tube is placed in the oven at 124±2° or in a bath at 90°, and the oxidation and titration are carried out as described on page 497.

Calculation

$0.0974\ (B - A)$ = milligrams of free cholesterol in sample analyzed.

B indicates the cubic centimeters of 0.1 N thiosulfate required to titrate the dichromate in a blank analysis, and A the cubic centimeters required to titrate in the analysis of the unknown.

The factor 0.0974 is the average found by Okey (32) in a series of analyses. It is 3.4 per cent above the theoretical factor, 0.0942, calculated from the formula of the digitonide as the amount oxidizable by 1 cc. of 0.1 N oxidizing agent. It appears that either the oxidation may not be quite complete to CO_2 and H_2O, or that a slight loss may occur in the precipitaton or washing.

Turner (40a) found that the error by the method was +2 to +4 per cent, while by the colorimetric method applied to Bloor's extract the error was +8 to +12 per cent.

Cholesterol esters can be calculated by subtracting *free cholesterol* from *total cholesterol.* The total cholesterol can be determined by any of the preceding colorimetric methods or, apparently with more accuracy, by applying the digitonin method to the saponified lipoid mixture of Stoddard and Drury.

GASOMETRIC LIPOID DETERMINATIONS

Backlin has shown that when the lipoids are oxidized with dichromate and sulfuric acid the carbon is quantitatively oxidized to CO_2, which can be determined gasometrically. This procedure can be substituted for Bloor's titration of excess dichromate, described above for lipoids and cholesterol digitonide. The gasometric method has over Bloor's titration of excess

dichromate the apparent advantage that spontaneous decomposition of some dichromate by heat does not affect the CO_2 yield, whereas it does affect the titration. Backlin claims greater immunity from error for his method, which is described on page 433 in the chapter on gasometric methods.

Iodometric titration of unsaturated fatty acids will be described in the appendix.

BIBLIOGRAPHY

1. Autenrieth, W., and Funk, A.: Ueber kolorimetrische Bestimmungsmethoden; Die Bestimmung des Gesamtcholesterins im Blut und in Organen. *Münch. med. Woch.*, 1913, 60, 1243.
2. Bang, I.: Verfahren zur titrimetrischen Mikrobestimmung der Lipoidstoffe. *Biochem. Z.*, 1918, **91**, 86; Die Mikrobestimmung der Blutlipoide, *Ibid.*, 235.
3. Baumann, E. J., and Holly, O. M.: The relation of lipoids to suprarenal physiology. I. The cholesterol and lipoid phosphorus contents of the blood of rabbits before and after suprarenalectomy. *J. Biol. Chem.*, 1923, **55**, 457.
4. Blix, G.: A critical study of the nephelometric determination of blood fat. *Skand. Arch. Physiol.*, 1924–25, **46**, 121.
5. Bloor, W. R.: On fat absorption. III. Changes in fat during absorption. *J. Biol. Chem.* 1913–14, **16**, 517.
   A method for the determination of fat in small amounts of blood. *J. Biol. Chem.*, 1914, **17**, 377.
6. Bloor, W. R.: Studies on blood fat. II. Fat absorption and the blood lipoids. *J. Biol. Chem.*, 1915, **23**, 317.
7. Bloor, W. R.: The determination of cholesterol in blood . *J. Biol. Chem.*, 1916, **24**, 227.
8. Bloor, W. R.: The determination of cholesterol in blood. *J. Biol. Chem.*, 1917, **29**, 437.
9. Bloor, W. R.: The determination of small amounts of lipid in blood plasma. *J. Biol. Chem.*, 1928, **77**, 53.
10. Bloor, W. R.: The oxidative determination of phospholipid (lecithin and cephalin) in blood and tissues. *J. Biol. Chem.*, 1929, **82**, 273.
11. Bloor, W. R., and Knudson, A.: The separate determination of cholesterol and cholesterol esters in small amounts of blood. *J. Biol. Chem.*, 1916, **27**, 106.
12. Bloor, W. R.; Pelkan, K. F., and Allen, D. M.: Determination of fatty acids (and cholesterol) in small amounts of blood plasma. *J. Biol. Chem.*, 1922, **52**, 191.
13. Clarke, H. T., and Rahrs, E. J.: A laboratory fractionating column. *J. Ind. Chem. Eng.*, 1923, **15**, 349.
14. Csonka, F. A.: A critique of certain data on the content of cholesterol and fatty substances in the blood, together with a modification of the colorimetric method for estimating cholesterol. *J. Biol. Chem.*, 1916, **24**, 431.
    A study of the nephelometric values of cholesterol and the higher fatty acids. *J. Biol. Chem.*, 1918, **34**, 577; 1920, **41**, 243.
15. de Toni, G. M.: The colorimetric estimation of cholesterol and lecithin in blood in connection with Folin and Wu's system of blood analysis. *J. Biol. Chem.*, 1926, **70**, 207.
16. Folin, O., and Wentworth, A. H.: A new method for the determination of fat and fatty acids in feces. *J. Biol. Chem.*, 1909–10, **7**, 421.

17. Fowweather, F. S.: The determination of the amount and the composition of the fat of faeces. I. Investigation of a "wet" method and comparison with the "dry" method. *Brit. J. Exp. Path.*, 1926, **7**, 7.
18. Gardner, J. A., and Fox, F. W.: A critical study of the methods of estimating cholesterol and its esters in tissues. Part II. *Biochem. J.*, 1924, **18**, 1058.
19. Grigaut, A.: Dosage colorimétrique de la cholestérine dans l'organisme. *Compt. rend. Soc. biol.*, 1910, **118**, 827.
20. Harnes, A. R.: The colorimetric determination of lipoid phosphorus in blood. *J. Biol. Chem.*, 1928, **77**, 405.
21. Holt, L. E.; Courtney, A. M., and Fales, H. L.: A method for the determination of fat in dried faeces and its distribution as soap, free fatty acids and neutral fat. An application to feces of the Roese-Gottlieb method for determining fat in dried and condensed milks. *Am. J. Dis. Child.*, 1919, **17**, 38.
22. Kumagawa, M., and Suto, K.: Ein neues Verfahren zur quantitativen Bestimmung des Fettes und der unverseifbaren Substanzen in tierischem Material nebst der Kritik einiger gebräuchlicher Methoden. *Biochem. Z.*, 1908, **8**, 212.
23. Leiboff, S. L.: A simplified method for cholesterol determination in blood. *J. Biol., Chem.*, 1924, **61**, 177.
24. Liebermann, C.: Ueber das Oxychinoterpen. *Ber. deut. chem. Ges.*, 1885, **18**, 1803.
25. Ling, S. M.: The determination of cholesterol in small amount of blood. *J. Biol. Chem.*, 1928, **76**, 361.
26. Luden, G.: Observation on cholesterin retention as a factor in cell proliferation. *J. Lab. Clin. Med.*, 1915–16, **1**, 662. Studies on cholesterol. III. The influence of bile derivatives in Bloor's cholesterol determination. Preliminary report. *J. Biol. Chem.*, 1917, **29**, 463. *J. Lab. Clin. Med.*, 1917–18, **3**, 93.
27. MacAdam, W., and Shiskin, C.: The cholesterol content of the blood in anaemia and its relation to splenic function. *Quart. J. Med.*, 1923, **16**, 193.
28. Meigs, E. B., and Marsh, H. L.: The comparative composition of human milk and of cow's milk. *J. Biol. Chem.*, 1913, **16**, 147.
29. Mueller, J. H.: A comparison of the results obtained by the colorimetric and gravimetric determinations of cholesterol. *J. Biol. Chem.*, 1916, **25**, 549.
A note on the solubility of cholesterol-digotonide. *J. Biol. Chem.*, 1917, **30**, 39.
30. Myers, V. C., and Wardell, E. L.: The colorimetric estimation of cholesterol in blood, with a note on the estimation of coprosterol in feces. *J. Biol. Chem.*, 1918, **36**, 157.
31. Nicloux, M.: Microdosage du carbone. Application à la détermination du carbone urinaire. *Bull. soc. chim. biol.*, 1927, **9**, 639.
32. Okey, R.: Adaptation of the blood oxidation procedure to the determination of small quantities of cholesterol as digitonid. *Proc. Soc. Exp. Biol. Med.*, 1929, **26**, 518.
33. Roese and Gottlieb: Roese-Gottlieb Method. Official and tentative methods of analysis of the Association of Official Agricultural Chemists. 1924, (Part XIX). p. 262, 2nd Edition, Washington; *Z. Nahr. Genussm.*, 1905, **9**, 531.
34. Salkowski, E.: Festschrift zu Ehren des sechszigsten Geburtstages, 1904.
35. Saxon, G. J.: A method for the determination of the total fats of undried feces and other moist masses. *J. Biol. Chem.*, 1914, **17**, 99.
36. Schmidt, H. B.: The clinical study of hypercholesterinemia. *Arch. Int. Med.*, 1914, **13**, 121.
A clinical study of cholesterinemia in patients with nitrogen retention. *Ann. Clin. Med.*, 1922, **1**, 66.

37. STEWART, C. P., AND WHITE, A. C.: The estimation of fat in blood. *Biochem. J.*, 1925, **19**, 840.

38. STODDARD, J. L., AND DRURY, P. E.: A titration method for blood fat. *J. Biol. Chem.*, 1929, **84**, 741.

39. SZENT-GYÖRGI, A.: Die gravimetrische Mikrochloresterinbestimmung. *Biochem. Z.*, 1923, **136**, 107.

40. SZENT-GYÖRGI, A.: Die titrimetrische Microcholesterinbestimmung. *Biochem. Z.*, 1923, **136**, 112.

40a. TURNER, M. E.: A simplification of the Okey method for the determination of cholesterol by oxidation of the digitonide. *J. Biol. Chem.*, 1931, **92**, 495.

41. WESTON, P. G.: A colorimetric test for cholesterol. *J. Med. Research*, 1912, **26**, 47.
WESTON, P. G., AND KENT, G. H.: Determination of the cholesterol content of human serum by the colorimetric method. *J. Med. Research*, 1912, **26**, 531.

42. WESTON, P. G.: Colorimetric methods for determining serum cholesterol. *J. Biol. Chem.*, 1917, **28**, 383.

43. WINDAUS, A. L.: Über die Entgiftung der Saponine durch Cholesterin. *Ber. deut. chem. Ges.*, 1909, **42**, 238.
Über die quantitative Bestimmung des Cholesterins und der Cholesterinesters in einigen normalen und pathologischen Nieren. *Z. physiol. Chem.*, 1910, **65**, 110.

CHAPTER X

TOTAL AND NON-PROTEIN NITROGEN

THE KJELDAHL METHOD

The procedures for the determination of nitrogen which are most extensively used at present are modifications of the method devised by Kjeldahl (17) in 1883. In this method material for analysis is digested in hot sulfuric acid; nitrogen is converted to ammonia which combines with the sulfuric acid to form ammonium sulfate. Kjeldahl's original procedure has been modified repeatedly to insure and to accelerate complete digestion. Among the expedients employed for this purpose are: 1, the addition of phosphoric acid or of potassium sulfate to raise the boiling point of the digestion mixture; 2, the addition of mercury, copper or other metals or their salts as catalysts; 3, the addition of oxidizing agents, such as hydrogen peroxide, perchlorate, and persulfate.

Although the Kjeldahl method is usually spoken of as a procedure for the determination of total nitrogen, it can be used for the estimation of nitrate nitrogen only if certain modifications are introduced, and fails to give quantitative results with many other nitrogen compounds (4). Such compounds, however, are not ordinarily encountered in the body and its secretions or excretions. Consequently for body fluids, tissues, and excretions the Kjeldahl method is really a total nitrogen determination.

The ammonia nitrogen in the digest is usually estimated by one of three methods. 1. The digest is rendered alkaline to liberate the ammonia, and the latter is distilled into standard acid and titrated. This is the universal procedure in macro analyses. In micro analyses both it and the two following are used. 2. Nessler's solution is added either to the original digestion mixture or to acid into which the ammonia has been distilled, and the ammonia is determined colorimetrically. 3. The digest is treated with hypobromite and the ammonia nitrogen is set free as nitrogen gas, which is measured.

KJELDAHL DIGESTION PROCEDURES FOR MACRO ANALYSES

Apparatus

Digestion is carried out in a round-bottomed 500 to 800 cc. flask of Pyrex, Jena or other hard glass which can resist hot alkali and extreme heat. The

flask has a long neck to prevent loss of material by spattering during the digestion.

Ordinarily the digestion is carried out under a hood. The flask is set over a Bunsen burner. The neck of the flask is inclined at an angle of about 30 degrees from the horizontal and the mouth is inserted into a hole in the side of a large lead pipe. The latter is connected directly with the flue of the hood and serves to carry off the fumes. The top of the burner should be close enough to the flask so that the flame can spread well over the round bottom of the flask. Setting the flasks at an angle insures against loss of material by spattering. Sulfuric acid condenses in the lead pipe which should, therefore, be slightly inclined upwards towards the flue and should

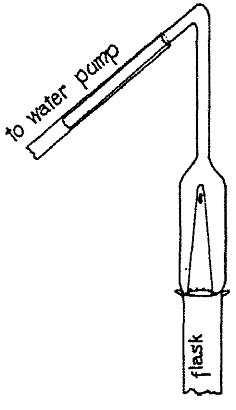


FIG. 69. Folin fume absorption tube (8)

have a small perforation at its lowest point, at the end furthest from the flue, to permit the condensed sulfuric acid to escape. This can be directed into a glass receiving vessel, and can be used for the preparation of cleaning fluid or other purposes for which pure acid is not required.

If a suitable hood or fume chamber is not available the sulfuric acid vapors may be carried away by suction. The outlet of a 2- or 3-liter wash bottle partly filled with caustic soda solution is connected with a suction pump; the inlet tube is connected with a Folin (8) fume absorption tube (see figure 69) or a small funnel. During the digestion the absorption tube is placed loosely over the mouth of the digestion flask and a constant current of air is drawn through. It is easy to devise a manifold which permits the removal of fumes from a whole series of flasks at the same time.

General procedure of macro digestion

Place enough material to contain 15 to 30 mg. of nitrogen in the Kjeldahl flask. Add a suitable amount of acid digestion mixture (see below). Place the flask on the burner as described above. To avoid excessive foaming or bumping only moderate heat is applied at first, especially if the material contains albuminous matter. A few bits of quartz, porous clay plate, or glass beads may be introduced into the flask to assist smooth boiling. As soon as vigorous boiling has commenced and initial excessive foaming and bumping have ceased the flame is increased. After the water present has been boiled off dense white fumes of sulfuric acid fill the flash and the organic matter becomes charred, making a black or brown mixture. Boiling is continued until the solution has become clear. If the charred material creeps up into or near the neck of the flask the latter is removed from the burner and the carbonaceous matter washed down either by rotating and tipping the flask or by the cautious addition of a few drops of concentrated sulfuric acid. (The flask may be handled with asbestos gloves or wooden tongs.) After the liquid has become clear the digestion in some cases is finished. In others gentle boiling with a lowered flame must be continued from one-half to two hours, depending on the nature of the material under analysis and the digestion mixture employed.

The flame is then removed and the flask is allowed to cool until water, when added cautiously, causes no sputtering. The contents are then diluted with about 300 cc. of water. If the flask is allowed to cool too long the digest may crystallize. It this occurs the crystals can be brought into solution by heating gently after water is added. The water used for dilution should be ammonia-free. Tap water from some sources may meet this requirement. Distilled water which has stood long exposed to laboratory air may contain significant amounts of ammonia.

If it is desired to interrupt the procedure at this point the flasks may be stoppered and set aside until it is convenient to continue the analysis.

Digestion mixtures

The Arnold-Gunning (2, 14) mixture consists of concentrated sulfuric acid, crystalline potassium sulfate, and copper sulfate or pure copper filings.

For digestion of the usual amounts of material about 20 cc. of sulfuric acid, 10 grams of potassium sulfate, and a small particle of copper or 0.1 to 0.2 gram of copper sulfate are used.

The digestion must be continued for about an hour after the solution has become clear. If it contains large amounts of protein, fat, or sugar even an hour's digestion and the addition of further acid will not always insure complete conversion of nitrogen to ammonia. The authors have found, for example, in the determination of serum proteins, that complete digestion within ninety minutes can be insured only by the addition of some extra oxidizing agent.

The use of hydrogen peroxide. Koch and McMeekin (16) have shown that by the use of 30 per cent hydrogen peroxide (Merck's superoxol or Kahlbaum's perhydrol) the digestion can be driven to completion more rapidly and certainly. The following procedure has been employed in the laboratory of one of the authors (P.) in the digestion of protein-containing solutions.

Digestion is begun with the sulfuric acid, potassium sulfate, copper sulfate method described above. At the end of thirty minutes, or when the solution is clear, the flask is removed from the flame and allowed to cool for fifteen to thirty minutes. Then 0.5 cc. of superoxol is carefully introduced with a pipette. Boiling is then resumed and continued thirty minutes after the reappearance of sulfuric acid fumes.

Thirty per cent hydrogen peroxide has one distinct advantage: it can be obtained nitrogen-free. Against this must be balanced the fact, that it is expensive and that it tends to etch the glass apparatus with which it comes in contact.

Davenport (5) has suggested the substitution of ten times the volume of the 3 per cent hydrogen peroxide solution of the United States Pharmacopœia. This is usually preserved with acetanilid, the nitrogen of which is quantitatively turned into ammonia by the digestion. If such peroxide is used a correction must be introduced for the acetanilid nitrogen. This according to Davenport, amounts to about 0.046 mg. per 1 cc. of peroxide used.

Dupray (7) has recommended a mixture of sulfuric, phosphoric and perchloric acids. Perchloric acid was suggested earlier as an aid to Kjeldahl digestion by Mears and Hussey (18). If, however, the organic material present does not reduce all the perchloric acid the latter destroys part of the ammonia formed. For this reason perchloric acid can not be used as a general reagent in Kjeldahl digestions.

Digestion with the aid of potassium persulfate, according to Wong (24). Wong has demonstrated that the period of digestion in the Arnold-Gunning modification of the Kjeldahl method can be reduced to a third by the addi-

tion of potassium persulfate. Persulfate had already been recommended by Huguet and Pitarelli, who added small amounts in the early part of the digestion. Wong, however, showed that the salt had a greater effect if it was added in the latter stage of the digestion and in the presence of moisture. If added before the greater part of the water has been boiled off the persulfate may be decomposed before the digest has become concentrated enough to destroy the organic matter.

To the usual amount of material add a small partical of copper or about 0.1 gram of copper sulfate, 5 grams of potassium sulfate, 20 cc. of concentrated sulfuric acid and a few bits of quartz or glass beads. Digest in the usual manner until the mixture becomes amber-colored (usually ten to thirty minutes, depending on the nature of the material which is being analyzed). At this point turn off the flame and allow the solution to cool for ten minutes. At the end of this period remove the flask from the shelf and, holding the flask with its mouth pointing away from the face, gradually and cautiously let about 3 cc. of water from a pipette dropper flow down the side of the flask, which is gently rotated at the same time. When this is done set the flask upright and dump into it from 3 to 10 grams (depending on the nature of the material under analysis) of potassium persulfate, taking care that as little powder as possible adheres to the sides of the neck. The persulfate used should be purified free of ammonia as described on p. 356. Mix the contents of the flask thoroughly by rotation and immediately resume heating. Continue heating until the acid digest becomes green. This usually takes about fifteen minutes. Cool the flask and dilute its contents in the usual manner.

The advantages of persulfate are that, unlike perchloric acid, it does not destroy any ammonia, it has not the explosive tendency of hydrogen peroxide, and does not injure glass.

DISTILLATION AND TITRATION OF AMMONIA

Apparatus used for distillation

In the type of apparatus usually employed for distillation of the ammonia a metal water bath with a continuous water flow cools a series of block tin or hard glass condenser tubes. Each of these is connected at the upper end by rubber tubing with a glass tube which passes just through a rubber stopper fitting tightly in the neck of the Kjeldahl flask. Beneath the flask is a burner.

The glass tube which connects flask and condenser is usually equipped with a safety bulb to prevent strong alkali from spattering into the condenser from the flask. Many types of such safety tubes are available on the market. That illustrated in figure 70 is one which has been found practical and efficient.

The lower end of the condenser is connected by rubber tubing to a straight glass tube, the lower end of which dips into the acid in the receiving flask or bottle. This glass tube should be of relatively wide bore to prevent fluid from sucking back into the condenser. It may be expanded at its upper end into a bulb to further provide against such a contingency.

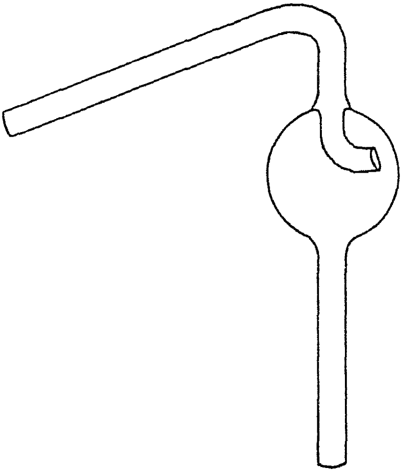


FIG. 70. Safety bulb for Kjeldahl digestion

For receiving the ammonia distillate wide-mouthed Erlenmeyer flasks are most convenient; but the ordinary pint or quart commercial milk bottles are cheap and quite satisfactory substitutes.

Reagents for distillation and titration

A concentrated solution of sodium hydroxide. This can be made by mixing equal weights of commercial solid NaOH and water. The mixture becomes hot, and should be made in earthen-ware or iron vessels, as it is likely to break large glass ones. Commercial caustic soda already in concentrated solution can be obtained on the market in steel drums. The impurities, chiefly iron, do not interfere with its use for Kjeldahl determinations. Those who are making many nitrogen studies will find it cheaper and safer

to purchase this solution than to make it. The volume of this alkali solution required to neutralize the acid used in a digestion is determined by rough titration.

Zinc, pieces of porous plate, powdered pumice, or talcum powder to prevent bumping. Powdered zinc is superior in promoting smooth boiling, but is the most expensive.

A 1 per cent solution of alizarin red.

Standard hydrochloric acid solution. The strength of this solution may be varied according to the amount of nitrogen expected. For most macro Kjeldahl work 0.1 or 1/14 N acid is employed. The 1/14 normal acid has the convenience that 1 cc. of it corresponds to 1 mg. of nitrogen, and calculation is simplified. In laboratories where Kjeldahl determinations do not constitute a large part of the usual work, however, it is hardly worth while to keep up the 1/14 N acid in addition to the 0.1 N used for general purposes.

Standard sodium hydroxide solution of the same strength as the standard acid.

Indicator. The indicator should change color on the acid side of neutrality, preferably at about pH 6 or a little lower. A 0.4 per cent solution of methyl red in methyl alcohol or a 1 per cent solution of alizarin red in water can be used. The analyst chooses the indicator to which his eye is most sensitive. Some analysts prefer the color change of a mixture of methyl red and para-nitrophenol. It is prepared as follows: dissolve 100 mg. of methyl red and 30 mg. of para-nitrophenol in 50 per cent alcohol with the aid of heat, and filter.

Procedure of distillation and titration

First the 0.1 or 1/14 N acid and the indicator are measured into the flask which is to receive the distillate, and the flask is put in place below the condenser, with the connecting tube from the condenser dipping just below the surface of the acid.

To the digest, diluted with about 300 cc. of water and cooled, one adds a piece of porous plate or pumice stone, or a little talcum powder or powdered zinc. A drop of two of alizarin red is also added. With the neck of the flask inclined at an angle, a measured volume of strong sodium hydroxide sufficient to somewhat more than neutralize the acid present is run in along the side of the flask. Care is taken not to get alkali on the part of the flask which comes into contact with the stopper during distillation. The heavy alkali solution runs under the acid solution in the flask without mixing, and forms a separate layer. The upper layer of solution remains acid, so that no ammonia can escape.

Without mixing its contents the flask is now connected with the still. After the connection has been made the contents are mixed by a whirling movement of the flask. The alizarin indicator should turn a deep red, showing that excess alkali is present. The burner under the flask is at once lighted. The heat of neutralization has warmed the solution already, so that distillation begins quickly after the burner is lighted. If the acid and alkali are not mixed before heat is applied boiling will

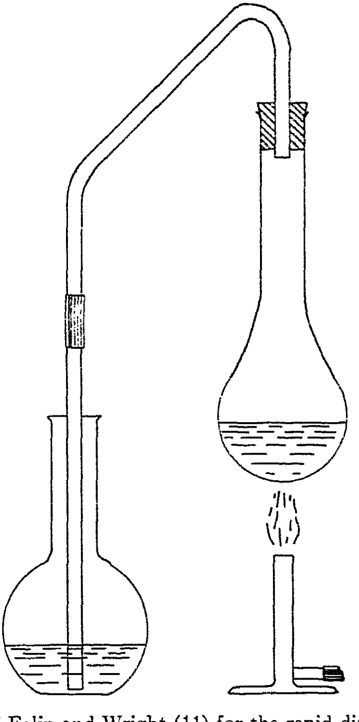


FIG. 71. Apparatus of Folin and Wright (11) for the rapid distillation of ammonia in the Kjeldahl procedure without the use of a condenser. For digestion a 300-cc. Kjeldahl flask is used and distillation is carried out over a micro-burner.

begin in an explosive manner which may drive the strong alkaline mixture into the condenser or even break the flask. If the acid and alkali were mixed before the flask is connected with the still, the alkalinity and the heat generated by the neutralization of the acid would cause immediate loss of ammonia.

During the first minutes of distillation only a low flame is used. As

distillation advances and the boiling point of the mixture rises the heat may be increased. Distillation is continued until from one-half to two-thirds of the solution has boiled away or until the first signs of bumping. Most of the ammonia comes over within the first few minutes. In the latter part of the distillation the delivery tube may be lifted out of the acid receiving solution as a precaution against sucking acid back into the condenser.

Folin and Wright (11) in their rapid distillation method without a condenser (see figure 71) allow the acid mixture to cool only four to five minutes after digestion is complete. Only 50 cc. of water is then added, followed by pumice and the requisite amount of sodium hydroxide. Acid and alkali are mixed and the mixture is vigorously distilled for only four or five minutes over a micro burner. The hot vapors pass directly into the standard acid in the receiving vessel, but no ammonia is lost if the delivery tube dips below the surface of the acid. In general, however, it is more certain and convenient to condense and cool the distillate before it enters the receiver.

When the distillation is complete the delivery glass tube is disconnected from the still before the flame is extinguished. If this order of procedure is reversed the receiving acid may be drawn back into the distillation flask before the latter can be detached. The delivery glass tube is removed from the receiving vessel and washed with a little distilled water.

The free acid in the vessel is titrated with standard alkali solution. The analysis is then finished.

If too little acid has been placed in the receiving flask to neutralize the ammonia, the receiving solution will change color in the course of distillation. If this change is noticed at once and a measured amount of extra acid is added, however, no ammonia is lost.

PRECAUTIONS

All reagents used for nitrogen determination should be as nearly as possible free from ammonia and other forms of nitrogen. All sources of ammonia fumes should be kept remote from the room where nitrogen determinations are being made.

Blank determinations must be made on the reagents at frequent intervals, because these reagents are likely to contain ammonia. If the blank amounts to more than 0.2 or 0.3 cc. of 0.1 N ammonia, it is desirable to find which reagent is responsible and replace it. It may be the potassium sulfate, the sulfuric acid, or the water used to dilute the digest.

If, by any chance, alkali or acid finds access to the condenser tubes, the

latter must be washed out thoroughly by distilling water through them from a weak acid solution until the distillate comes through neutral. If a condenser has stood for some days unused, it is a wise precaution to wash it thus before it is used in an analysis.

During both digestion and distillation the analyst should have his eyes protected by glasses (see p. 48).

Calculations

In titration by the reaction

$$NH_4OH + HCl = NH_4Cl + H_2O$$

one milligram atom, or 14 mg., of ammonia nitrogen, is titrated by 1 mg. equivalent, or 1 cc. of 1 N acid. Hence the cubic centimeters of 1 N acid neutralized by the ammonia are multiplied by 14 to calculate milligrams of nitrogen.

The calculation formula is

$$\text{Milligrams N in sample} = 14\ F\ (A - B - C).$$

A = cubic centimeters of standard acid, 0.1 or 1/14 N, placed in receiver.

B = cubic centimeters of standard alkali of same strength required to titrate the excess of acid not neutralized by ammonia.

C = value of $A - B$ found in blank analysis of the reagents.

F = strength of standard alkali (0.1 or 1/14) in terms of normality.

MICRO MODIFICATIONS OF THE KJELDAHL METHOD

Although Strauss (21) is credited by Folin with having made the first important and extensive studies of the non-protein nitrogen of the blood in health and disease, it is the name of Folin (9) himself which is most intimately connected with the development of the subject and of the methods for the determination of minute amounts of nitrogen.

The term micro-method is applied generally, in the case of nitrogen, to procedures that permit reasonably accurate determination of as little as 1 mg. of nitrogen or less. Methods adapted to the estimation of even smaller amounts (6, 19, 22) such as the non-protein nitrogen in 0.1 to 0.2 cc. of finger blood, have been proposed but have not been widely employed.

The procedures are similar to those employed in the macro-Kjeldahl method, with modification to permit the use of small quantities of material. The filtrate is first digested with a suitable digestion mixture. The ammonia may then be distilled into acid of appropriate strength and titrated with alkali or it may be determined in the digest, either colorimetrically after treatment with Nessler's reagent, or gasometrically by treatment with

hypobromite. Removal of the ammonia by aeration has been recommended; but it is more time consuming than distillation and has not given as constant results in the hands of most workers who have compared the two procedures. Direct nesslerization and colorimetric determination of the ammonia in the digest make for economy of time and material. If more than 1 mg. of nitrogen is determined, however, distillation and titration is more exact, and is preferred to the colorimetric procedure by many, including the authors. For gasometry (23) the digest is washed directly into the manometric gas apparatus and the ammonia is determined by measurement of the nitrogen gas evolved after addition of hypobromite. This procedure avoids distillation and determines the ammonia with less than 1 per cent error.

The percentage error in the micro methods is likely to be greater than in macro Kjeldahl analyses. Impurities in the reagents affect the micro results more seriously, and there is more liability to error arising from any deviations from perfect technique in digestion, distillation, or titration. The digestion in particular demands attention. Directions for use of each digestion mixture must be followed precisely. Peculiarly enough, if one takes 1/20th as much of both reagents and digestion mixture one does not get necessarily the same complete yield of ammonia that is obtained with the macro method. In general the macro rather than the micro method is to be recommended when sufficient material is available and one desires to keep the error below 1 per cent.

Nevertheless if the micro analysis is performed with the precautions detailed below, with steam distillation followed by titration from a calibrated micro burette, or with gasometric determination of the ammonia, it is possible for a practiced analyst to approach in micro analyses the accuracy of the macro Kjeldahl.

MICRO KJELDAHL DIGESTION BY THE ARNOLD-GUNNING METHOD, WITH AND WITHOUT PEROXIDE

For protein-free fluids, such as non-albuminous urine and blood filtrates, the sulfuric acid and copper sulfate mixture of the Arnold-Gunning method applied to micro analyses gives satisfactory results. If protein is present, however, an extra oxidizing agent must be added. As such hydrogen peroxide, introduced by Koch and McMeekin (16), may be used.

The digest obtained is suitable for distillation and titration of the ammonia. It is not suitable for direct nesslerization nor for the gasometric determination of the ammonia, both of which are interfered with by the copper present.

Procedure

Material containing 0.3 to 4 mg. of nitrogen is placed in a 100-cc. Kjeldahl flask with 1 cc. of concentrated sulfuric acid, 5 or 6 drops of 5 per cent copper sulfate solution, and 0.5 gram of potassium sulfate. One or two glass beads are added to prevent bumping, and the mixture is heated until the water has been driven off and the concentrated residue has become clear. Heating is then continued two minutes longer. This finishes the digestion if the material contains no protein or lipoids.

If protein is present the cleared digest is cooled and 1 or 2 drops of 30 per cent hydrogen peroxide are added. Then the mixture is heated to boiling for one minute. Boiling may commence explosively. It is less likely to do so if the tube is agitated by tapping until boiling begins.

It is probable that the hydrogen peroxide in the Arnold-Gunning micro digestion could be advantageously replaced by potassium persulfate used as in the procedure next described.

MICRO KJELDAHL DIGESTION WITH THE SULFURIC-PHOSPHORIC-PERSULFATE MIXTURE OF VAN SLYKE (23)

In this procedure phosphoric acid is used instead of copper to accelerate the first stages of the digestion, and persulfate is used to complete the oxidation. Digestion with this mixture is rapid, and is complete as soon as the solution clears, whether proteins are present or not.

The digest obtained is suitable for determination of the ammonia by distillation and titration, or by the gasometric hypobromite method. It is not recommended for the direct nesslerization because the phosphoric acid is likely to detach particles of silica from the glass so that the solution is not entirely clear for colorimetric readings.

The reagents, ammonia-free potassium persulfate and a 3:1 mixture of concentrated sulfuric and phosphoric acids, are described on pages 354–56, in the chapter on gasometric methods, and the digestion procedure on page 356.

MICRO KJELDAHL DIGESTION FOR DIRECT NESSLERIZATION BY THE PERSULFATE METHOD OF WONG (25)

The determination of ammonia formed in Kjeldahl digestion by adding Nessler's solution directly to the diluted digest, and thus avoiding distillation, was introduced by Folin and Denis (11). These authors, however, used for digestion a mixture consisting chiefly of phosphoric acid, 3 parts to 1 of sulfuric, which was likely to detach silica from the digestion tubes and

cloud the solution. This difficulty was overcome by Wong's substitution of potassium persulfate in place of the phosphoric acid as accelerator of the digestion.

Reagents

Fifty per cent sulfuric acid by volume. Mixture of equal volumes of concentrated sulfuric acid and water.

Ammonia-free potassium persulfate, saturated solution. The potassium persulfate is purified free of ammonia, if necessary, by the process described on page 356. Of the ammonia-free salt 7 or 8 grams are shaken in a glass stoppered vessel with 100 cc. of water. The undissolved crystals are left in the flask, and serve to keep the solution saturated, even if some of the dissolved persulfate undergoes decomposition.

Digestion

To material containing 0.2 to 0.3 mg. of nitrogen add 1 cc. of the 50 per cent sulfuric acid and two or three glass beads. Digest as usual until the water has been driven off and white sulfuric acid fumes appear. When the tube or flask is nearly full of fumes, cover it with a small watch-glass and reduce the flame so that the acid mixture boils gently. Continue the gentle boiling for two to four minutes.[1] Remove the burner and allow the solution to cool one minute. Take off the watch-glass and add with a fine pipette or dropper, 2 drops[1] to 1 cc. of saturated persulfate solution. Replace the burner and continue boiling until the digestion mixture becomes colorless, which is usually fifteen seconds to two minutes[1] after the reappearance of sulfuric acid fumes.

DISTILLATION AND TITRATION IN MICRO KJELDAHL ANALYSES. BOCK AND BENEDICT'S DISTILLATION (3)

A simple form of apparatus similar to that employed by Bock and Benedict is illustrated in figure 72.

[1] The time of boiling and the quantity of persulfate depend on the nature of the material which is being analyzed. Wong prescribes the following boiling periods and quantities of persulfate for different purposes.

	PRELIMINARY DIGESTION; MINUTES AFTER WHITE FUMES APPEAR	QUANTITY OF SATURATED POTASSIUM PERSULFATE	FINAL DIGESTION; MINUTES REQUIRED TO CLEAR SOLUTION AFTER WHITE FUMES REAPPEAR
Urine	2	2 drops	$\frac{1}{4}$
Blood	3	0.5 cc.	—
Milk	4	1.0 cc.	2

Into the receiving flask, F, is measured sufficient 0.01 or 0.02 N hydrochloric acid to neutralize the ammonia expected in the distillate, and 4 or 5 drops of 0.1 per cent alcoholic methyl red, and enough water to bring the total volume to about 20 cc. Flask F is placed in position as shown in figure 72, and the apparatus is completely set up except for Flask A. The pinch-cock on tube B is then released and by means of suction enough concentrated sodium hydroxide is drawn into the tube

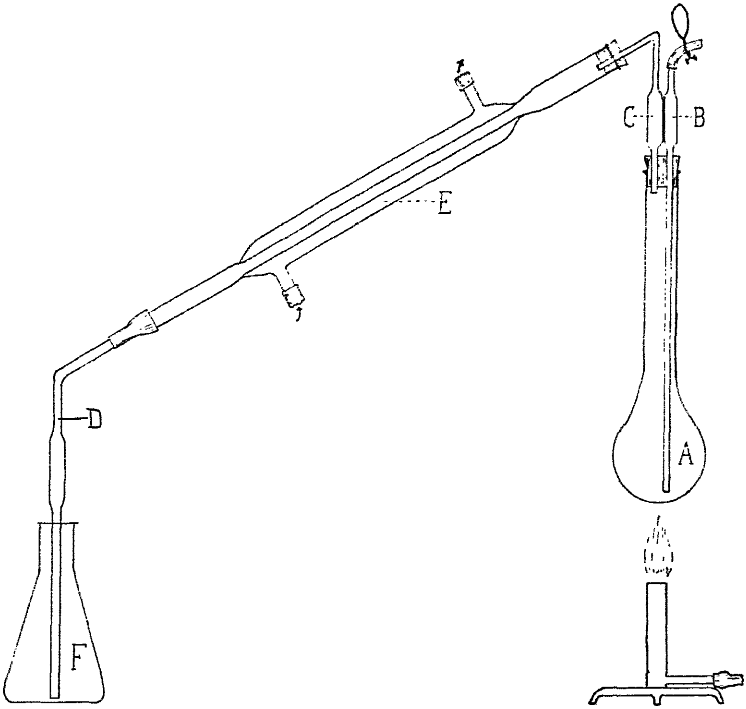


FIG. 72. Apparatus for micro-Kjeldahl distillation, after Bock and Benedict (3). A micro-Kjeldahl flask; B, tube for introduction of alkali; C, safety bulb; D, delivery tube with safety bulb; E, 6-inch condenser; F, receiving flask containing acid and indicator.

to neutralize the acid in the digestion mixture. The pinch-cock is then again closed so that the fluid is retained in tube B. (A long rubber tube with a glass connection must be interposed between the mouth and the rubber tube at the top of B, when the latter is filled with alkali, to avoid drawing the alkali into the mouth.)

Flask A, containing the digest diluted with 7 to 10 cc. of water and

cooled, is put in place as shown in the figure. The pinch-cock on *B* is now opened and the alkali is allowed rapidly to enter the flask, where it is immediately mixed with the acid mixture by blowing into the rubber tube attached to *B*. The pinch-cock is then again closed and the flame from a micro-burner is applied to the flask. The flame must be well protected from draughts or air currents. The solution is boiled vigorously for two minutes, or until it bumps. As soon as distillation is complete the pinch-cock is opened (this measure can also be resorted to if the fluid in *F* begins to suck back into the condenser earlier), the flame is extinguished or removed, and the upper end of the condenser is disconnected. The condenser and tube *D* are then rinsed into the receiving flask with distilled water, and the acid is titrated with 0.01 or 0.02 N sodium hydroxide.

Calculation is by the same formula given on page 525 for macro Kjeldahl analyses.

The standard acid and alkali used may be 0.01, 0.02, or 1/70 N. The N/70 has the convenience that each cubic centimeter represents 0.2 mg. of nitrogen. The more dilute the alkali is the more difficult it is to keep it from changing its titration value. The latter increases quite rapidly from contact with glass. A satisfactory procedure is to use the 0.02 or 1/70 N solutions, measuring the acid with a calibrated Ostwald pipette, 3, 5, or 10 cc., according to the amount of nitrogen expected, and titrating with alkali from a Bang micro burette (fig. 1, p. 13), by which one can obtain an accuracy of 0.02 cc.

The standard alkali, even if kept in paraffined bottles, must be checked by titration against the acid every few days. It is also necessary at frequent intervals to run blank analyses on the reagents, because they can neither be obtained nor kept entirely free from ammonia.

Pregl's (20) steam distillation as modified by Walther F. Goebel (personal communication)

Pregl has shown that bumping can be prevented and distillation hastened by passing steam through the solution in the Kjeldahl flask during the procedure. An apparatus for this purpose is shown in figure 73. The use of steam distillation makes the process so much more smooth, safe and certain, that it is well worth while to assemble the apparatus in figure 73 if any considerable number of analyses is anticipated.

A Pyrex 2-liter Erlenmeyer flask *a* serves as a steam generator. An inverted 200-cc. flask, *b*, stops condensed water from running into the Kjeldahl

flask. The rubber connecting tube, *c*, should be boiled out with dilute alkali before it is used for the first time. A T-tube, *d*, connects the steam generator flask with the Kjeldahl flask. To the upright arm is connected, by means of rubber tubing controlled with a pinch-cock, a 50-cc. burette containing concentrated alkali.

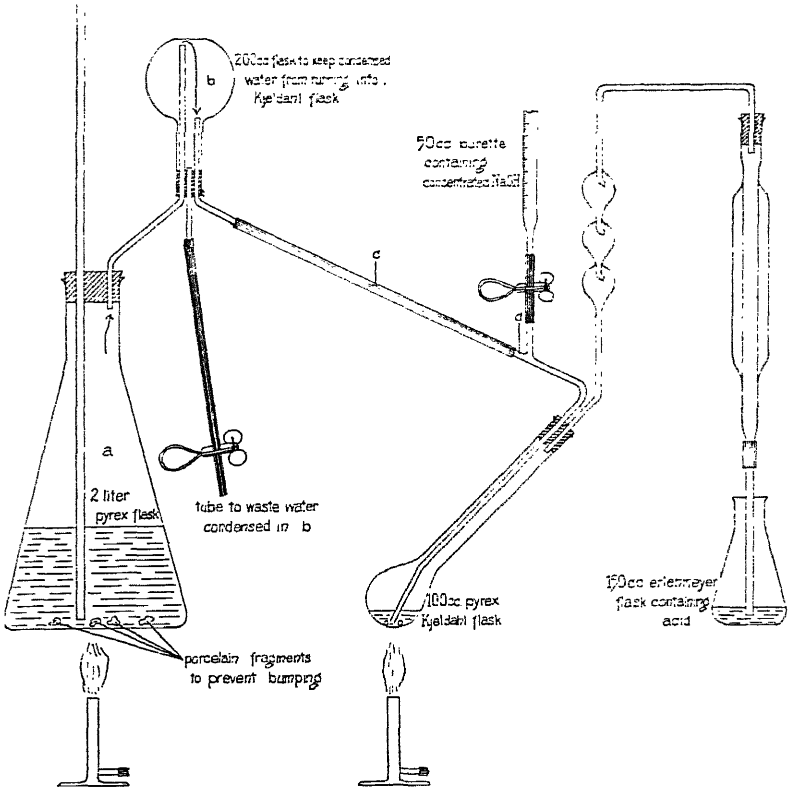


FIG. 73. Walther Goebel's modification of Pregl's (20) apparatus for micro Kjeldahl distillation. For details of use see text.

Procedure

With steam generator *a* disconnected from *d* the water in the generator is heated to boiling (it is well to place a few bits of fresh porous plate in *a* to promote smooth boiling). The Kjeldahl flask is connected with the condenser and alkali burette as shown in the figure, but not yet with *c*. The necessary amount of alkali is admitted to the Kjeldahl

flask from the burette, whereupon connection with the steam generator through *c* and *d* is made immediately. The micro-burner is then placed under the Kjeldahl flask and the contents of the latter are boiled for three minutes.

Titration is performed as described above for the Bock-Benedict distillation.

DIRECT NESSLERIZATION. FOLIN AND DENIS (10) AND WONG (25)

Ammonia forms with Nessler's solution a yellow-red compound. If the ammonia exceeds a certain concentration the compound precipitates at once. But if conditions, particularly the dilution, are probably chosen Folin and Denis have shown that the colored compound remains in solution long enough to be determined colorimetrically. The error involved in the colorimetry can, in the experience of the authors, amount to 2 or 3 per cent. Consequently colorimetric determination is less accurate than distillation and titration when more than 1 mg. of nitrogen is determined. The colorimetric determination can, however, be performed with maximum accuracy when as little as 0.2 mg. of nitrogen is present. This is the case in the *determination of non-protein* nitrogen of blood, except in rare instances of gross nitrogen retention. In 1:10 blood filtrates the 5 or 10-cc. portions convenient for micro analyses contain, in the case of normal blood, an average of only about 0.17 and 0.34 mg. respectively of nitrogen. For such small amounts the colorimetric or gasometric methods are preferable to distillation and titration.

Preparation of Nessler's solution according to Koch and McMeekin (16)

This reagent is made with precautions to avoid formation of mercurous salts. Koch and McMeekin state that it is an improvement over previous solutions in that it never separates a precipitate of dark green mercurous compounds and is less likely to cause turbidity when added to ammonia solutions.

Dissolve 22.5 grams of iodine in 20 cc. of water containing 30 grams of potassium iodide. After the solution is complete add 30 grams of pure metallic mercury, and shake the mixture well, keeping it from becoming *hot* by immersing in tap water from time to time. Continue this until the supernatant liquid has lost all of the yellow color due to iodine. Decant the supernatant aqueous solution and test a portion by adding a few drops thereof to 1 cc. of a 1 per cent soluble starch solution. Unless the starch test for iodine is obtained the solution may contain mercurous compounds. If the test is negative add a few drops at a time an iodine solution of the same concentration as employed above, until a faint excess of free iodine can be detected by adding a few drops to 1 cc. of the starch solution. Dilute to 200 cc. and mix well.

To 975 cc. of an accurately prepared 10 per cent sodium hydroxide solution now add the entire solution of potassium mercuric iodide prepared above. Mix thoroughly and allow to clear by standing.

This solution is to be used in the proportion of 10 cc. per 100 cc. of nesslerized solution.

Nessler's solution with extra alkali. One volume of the above Nessler's solution is mixed with 2 volumes of 10 per cent NaOH. The extra alkali serves in this analysis to neutralize the sulfuric acid of the digest.

Standard ammonia solution. A convenient standard solution, containing 1 mg. of nitrogen in 10 cc. can be prepared by diluting 0.3821 gram of ammonium chloride or 0.472 gram of ammonium sulfate to 1000 cc.

Procedure

Digestion is carried out by Wong's procedure (25) as described above in a 25 by 200 mm. tube marked at 35 and 50 cc. The sample taken for analysis should contain preferably 0.2 to 0.3 mg. of nitrgoen.

Nesslerization. When digestion is completed the digestion tube is allowed to cool for one to two minutes, then its contents are diluted with 15 to 25 cc. of water. It is then cooled further, approximately to room temperature, and made up to 35 cc. with water.

In a similar test tube marked to contain 35 and 50 cc. place a volume of the standard ammonia solution which will contain an amount of nitrogen comparable to that expected in the unknown; e.g., if 5 cc. of 1:10 blood filtrate are analysed, one uses 2 cc. of the standard ammonia solution with 0.2 mg. of ammonia nitrogen. One cubic centimeter of 50 per cent sulfuric acid from the same lot used in the digestion is added, and enough water to dilute to 35 cc.

Now to both unknown and standard add 15 cc. of "Nessler's solution with extra alkali," using an ordinary bulb pipette of large aperture. Let the Nessler's solution fall directly into the digest solution, without first touching the wall of the test tube. Close each tube with a clean rubber stopper, mix, and compare in a colorimeter.

Calculation

$$A \times \frac{S}{U} = \text{milligrams of nitrogen in sample analysed.}$$

S and U represent the depths of layer of solution of standard and unknown read on the colorimeter scale, and A represents the number of milligrams of ammonia nitrogen in the standard.

In this, as in other micro-analyses, a blank analysis must be performed on the reagents, and the amount of nitrogen found subtracted from that calculated by the above formula.

GASOMETRIC METHOD OF VAN SLYKE (23)

An accurate gasometric technique for the micro Kjeldahl determination of from 0.2 to 1.5 mg. of nitrogen is described in the chapter on gasometric analysis, page 354.

ANALYSIS OF URINE FOR TOTAL AND NON-PROTEIN NITROGEN BY THE MACRO KJELDAHL METHOD

The method of choice for the determination of nitrogen in urine will depend upon: 1, whether the urine contains protein in significant quantites; 2, whether in an albuminous urine it is desired to determine the total nitrogen or only the non-protein nitrogen; 3, whether information concerning the quantity and character of the protein in albuminous urine is also desired.

1. *If the urine contains no albumin* its total nitrogen can be determined by any one of the macro-procedures described in the preceding section.

Usually 5 cc. of urine is used for an analysis. The ammonia is received in from 30 to 50 cc. of 0.1 N hydrochloric acid, and is titrated with 0.1 N sodium hydroxide.

If the urine is extremely concentrated a smaller amount may be taken for analysis or the urine may be diluted to a specific gravity of about 1.020 before a sample is taken.

2. *If the urine contains protein, but knowledge of its total nitrogen content alone* is desired the same quantities of urine and reagents are employed, but one of the digestion procedures which insures rapid digestion of protein is used. These are: Arnold-Gunning + 30 per cent hydrogen peroxide or Wong's potassium persulfate method.

3. *If the urine contains protein, but knowledge of its non-protein nitrogen content alone is desired* procedure 1 may be applied to a suitable aliquot of the protein-free filtrate of urine. The protein precipitants most frequently employed for the production of such protein-free filtrates are trichloroacetic acid and tungstic acid.

Precipitation of urine protein with trichloracetic acid. **Twenty-five cubic centimeters of urine in a large centrifuge tube or a small Erlenmeyer flask are mixed with an equal volume of 10 per cent trichloroacetic acid and stood in a boiling water bath long enough to bring the contents of the vessel almost to the temperature of the bath. The liquid is then filtered through a dry filter or centrifuged.**

After centrifuging if the supernatant liquid is not clear, add to the tube 0.5 to 1.0 gram of dry sodium chloride, stir up the precipitate, heat as before, and centrifuge again.

If filtration is used instead of centrifuging, and the filtrate, after the first portions have been returned to the filter, is not clear, treat another 25 cc. of urine with 25 cc. of trichloroacetic acid and 0.5 to 1.0 gram of dry sodium chloride, heat as before in the boiling water bath, and filter.

Ten cubic centimeter portions of protein-free filtrate or supernatant liquid are analysed by the simple Arnold-Gunning method.

Precipitation of urine protein with tungstic acid (27). To 5 parts of urine add 1 part of 10 per cent sodium tungstate and 1 part of $\frac{2}{3}$ N sulfuric acid. Mix thoroughly. Centrifuge the solution. If the supernatant liquid is not clear add a few more drops of $\frac{2}{3}$ N sulfuric acid, stir up the precipitate again and recentrifuge. Use 5 cc. aliquots of the clear, protein-free, supernatant liquid for the determination of nitrogen by Precedure 1 above.

4. *If the urine contains protein and knowledge of both its non-protein and its protein nitrogen content is desired.* One of the special methods for the determination of urine proteins (see chapter on serum and urine proteins) is used to determine the protein nitrogen. The non-protein nitrogen is determined by Procedure 3, above.

TOTAL NITROGEN IN FECES

Feces, collected in the usual manner over a given period (see chapter on treatment of biological material), must be preserved and treated in such a way that ammonia does not escape before analysis. All methods that involve drying feces cause loss of ammonia. Hawk (15) advises preserving the stools with thymol at ice-box temperature. The authors prefer collecting the feces in sulfuric acid as described on page 78.

An amount of fecal matter containing about 20 to 30 mg. of nitrogen (an adult usually excretes about 1 gram of nitrogen daily in the feces) is weighed out and transferred to a Kjeldahl flask. It is digested by the Arnold-Gunning + peroxide or Wong's potassium persulfate technique. Account is taken of the sulfuric acid which has already been used as a preservative for the stools and correspondingly less is added with the digestion mixture. The ammonia is distilled into 30 to 50 cc. of 0.1 N hydrochloric acid and titrated with 0.1 N hydroxide.

TOTAL NITROGEN IN BLOOD CELLS, TISSUES, BLOOD, SERUM AND OTHER BODY FLUIDS

The total nitrogen of tissues, bloods, serum or tissue fluids can usually be determined by subjecting a suitable quantity of the material to the macro-Kjeldahl procedure, using for digestion the Arnold-Gunning mixture + peroxide (p. 519), or Wong's persulfate technique (p. 519).

Table 54 indicates the size of samples and strength of acid and alkali required for analysis of various biological materials.

TABLE 54

Size of Samples for Kjeldahl Analyses

	Approximate usual concentration of nitrogen	Size of sample to give 20 to 30 mg. N for macro-Kjeldahl	Size of sample to give 1 to 1.5 mg. N for distillation or gasometric micro-Kjeldahl	Size of sample to give 0.2 to 0.3 mg. N for direct Nesslerization or gasometric micro-Kjeldahl
	per cent			*grams or cc.*
Blood cells	5	0.5	0.025	0.005
Whole blood	3	1.0	0.05	0.01
Serum or plasma	1	2	0.1	0.02
Exudates and transudates	0.5	5	0.2	0.05
Spinal fluid, protein-free edema fluid	0.05	50	2	0.5
1:10 blood filtrate	0.004		20	5

NON-PROTEIN NITROGEN IN BLOOD, SERUM OR BODY FLUIDS

The term "non-protein nitrogen" of the blood in its strictest interpretation includes all the nitrogen-containing substances of blood that are not proteins. However, it is commonly used to refer to the nitrogen containing substances that remain in the filtrate after the proteins have been precipitated. Urea, ammonia, uric acid, creatine, creatinine and amino acids, according to Wu (26) constitute nearly the entire non-protein nitrogen of plasma, but in the cells a considerable part of the non-protein nitrogenous substances are of unknown nature. Hence the determination has a less definite significance when performed, as usual, on whole blood, than when plasma is used.

When precipitants other than alcohol are used to remove proteins the nitrogenous lipoids are carried down with the protein coagulum. Lipoid nitrogen, therefore, does not form part of the fraction ordinarily determined as "non-protein." A better name for the latter would be "non-colloid" or

"crystalloid" nitrogen. The term non-protein nitrogen, commonly abbreviated to n.p.n. has, however, apparently become permanent.

Many methods have been proposed for the quantitative determination of the non-protein nitrogen, but all involve the same principles: first, the removal of the protein by some suitable precipitant; second, the analysis of the filtrate for nitrogen by some modification of the Kjeldahl method. Numerous protein precipitants have been used, with more or less satisfactory results, and most of them have been discarded because of certain unfavorable properties or inconveniences. The properties and peculiarities of the these precipitants have been considered under the general heading "Precipitation of proteins" on page 63. The two precipitants that are now most generally employed in the determination of blood non-protein nitrogen are trichloracetic acid and tungstic acid.

For non-protein nitrogen tungstic acid and trichloroacetic acid filtrates yield practically identical results. The choice of precipitant depends largely on the other uses for which the filtrate is intended. Which of the two precipitants shall be used when only non-protein nitrogen is to be determined is merely a matter of convenience. Trichloracetic acid has a slight advantage in this respect, because the reagent is simpler, cheaper to prepare, filters somewhat faster from the protein coagulum, and yields a larger volume of filtrate.

BIBLIOGRAPHY

1. Allen, E. R., and Davisson, B. S.: On the relative accuracy of colorimetric and titrimetric procedures for determining nitrogen as ammonia. *J. Biol. Chem.*, 1919, **40**, 183.
2. Arnold, C., and Wedemeyer, K.: Beiträge zur Stickstoffbestimmung nach Kjeldahl. *Z. anal. Chem.*, 1892, **31**, 525.
3. Bock, J. C., and Benedict, S. R.: An examination of the Folin-Farmer method for the colorimetric estimation of nitrogen. *J. Biol. Chem.*, 1915, **20**, 47.
4. Dakin, H. D., and Dudley, H. W.: Some limitations of the Kjeldahl method. *J. Biol. Chem.*, 1914, **17**, 275.
5. Davenport, H. A.: A note on the Koch and McMeekin method for the determination of nitrogen; with special reference to the non-protein nitrogen of blood and urine. *J. Lab. Clin. Med.*, 1926–27, **12**, 286.
6. Donald, R.: A simple rapid method of estimating the filtrate nitrogen in small quantities of blood and of other body fluids. *Quart. J. Med.*, 1917–18, **11**, 19.
7. Dupray, M.: A modified digestion acid for non-protein nitrogen determination. *J. Lab. Clin. Med.*, 1926–27, **12**, 387.
8. Folin, O., and Denis, W.: An apparatus for the absorption of fumes. *J. Biol. Chem.*, 1912, **11**, 503.
9. Folin, O., and Denis, W.: New methods for the determination of total non-protein nitrogen, urea, and ammonia in blood. *J. Biol. Chem.*, 1912, **11**, 527.

10. FOLIN, O., AND DENIS, W.: Nitrogen determinations by direct nesslerization. I. Total nitrogen in urine. *J. Biol. Chem.*, 1916, **26**, 473.
11. FOLIN, O., AND WRIGHT, L. E.: A simplified macro-Kjeldahl method for urine. *J. Biol. Chem.*, 1919, **38**, 461.
12. FOLIN, O., AND WU, H.: A system of blood analysis. *J. Biol. Chem.*, 1919, **38**, 81.
13. GREENWALD, I.: The estimation of non-protein nitrogen in blood. *J. Biol. Chem.*, 1915, **21**, 61.
14. GUNNING, J. W.: Ueber eine Modification der Kjeldahl-Methode. *Z. anal. Chem.*, 1889, **28**, 188.
15. HAWK, P. B.: Practical Physiological Chemistry. 8th ed., 1923. P. Blakiston's Son and Company, Philadelphia.
16. KOCH, F. C., AND MCMEEKIN, T. L.: A new direct nesslerization micro Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. *J. Am. Chem. Soc.*, 1924, **46**, 2066.
17. KJELDAHL, J.: Neue Methode zur Bestimmung der Stickstoffs in organischen Körpern. *Z. anal. Chem.*, 1883, **22**, 366.
18. MEARS, B., AND HUSSEY, R. E.: The use of perchloric acid as an aid to digestion in the Kjeldahl nitrogen determination. *J. Ind. Eng. Chem.*, 1921, **13**, 1054.
19. PETERS, A. W.: The micro-determination of nitrogen by direct nesslerization and of total solids, in drop quantities of human blood. *J. Biol. Chem.*, 1919, **39**, 285.
20. PREGL, F.: Die quantitative organische Mikroanalyze. Berlin, 1923. 2nd. Edition.
21. STRAUSS, H.: Die chronischen Nierenentzündungen in ihrer Einwirkung auf die Blutflüssigkeit und deren Behandlung. A. Hirschwald, Berlin, 1902.
22. TAYLOR, A. E., AND HULTON, F.: On the estimation of non-protein nitrogen and glucose in finger blood. *J. Biol. Chem.*, 1915, **22**, 63.
23. VAN SLYKE, D. D.: Gasometric micro-Kjeldahl determination of nitrogen. *J. Biol. Chem.*, 1927, **71**, 235.
24. WONG, S. Y.: The use of persulfate in the estimation of nitrogen by the Arnold-Gunning modification of Kjeldahl's method. *J. Biol. Chem.*, 1923, **55**, 427.
25. WONG, S. Y.: The use of persulfate in the estimation of nitrogen by Folin's direct nesslerization method. *J. Biol. Chem.*, 1923, **55**, 431.
26. WU, H.: Separate analyses of the corpuscles and the plasma. *J. Biol. Chem.*, 1921, **51**, 21.
27. WU, H., AND LING, S. M.: Colorimetric determination of proteins in plasma, cerebrospinal fluid and urine. *Chinese J. Physiol.*, 1927, **1**, 161.

CHAPTER XI

UREA

GENERAL PRINCIPLES OF ANALYTICAL METHODS

Urea is capable of a number of reactions that have been used for its quantitative estimation. Of these only two, precipitation with xanthydrol, and decomposition with the enzyme, urease, into ammonia and carbon dioxide, give quantitative results with urea and entirely negative results with the other constituents of blood and urine. However, three less specific reactions, those with heat, hypobromite, and mercuric salts, have formerly enjoyed general use, and have provided methods of analysis that are sufficiently close approximations to be still used for certain purposes. These procedures will accordingly be briefly considered in the following discussion.

Urease

Urease is an enzyme which decomposes urea quantitatively into ammonium carbonate according to the reaction

$$CO\ (NH_2)_2 + 2\ H_2O = (NH_4)_2\ CO_3.$$

For analytical purposes urea may be estimated by determining either the ammonia or the carbon dioxide formed by the above reaction.

The enzyme was discovered in soy beans by Takeuchi (42) but has been found to be several times as abundant in sword beans (4) and jack beans (33).

The mode of action of urease was first studied after Takeuchi by H. E. and E. F. Armstrong, Horton and Benjamin, (5, 6, 7) who showed that the action was retarded by alkalies or mineral acids, but was accelerated by the weak acids, hydrocyanic and carbonic (because of the buffer effect of their salts (48)). These authors and Takeuchi also demonstrated the extreme specificity of the enzyme for urea: it attacked no other nitrogenous compounds, among many tested, not even one so closely related as monomethyl urea. It is this extreme specificity which makes it possible to use the enzyme directly for quantitative urea estimation in so complex a mixture as blood.

Marshall (32) showed that urease obeys the general enzymic law, that rate of action is proportional to enzyme concentration; he developed the first methods for use of the enzyme in blood and urine analyses.

Van Slyke and Cullen (45) by means of acetone precipitation of soy bean

extracts (the more active jack beans are now used) prepared the enzyme in the form of a dry soluble powder which maintained its activity indefinitely. With this preparation Van Slyke and Cullen (47) and Zacharias (48) made a detailed study of the kinetics of the enzyme's action. They showed that the rate of action is doubled by every 10° increase in temperature from 0° to 55°, above which it begins to be destroyed; that the optimum pH is at approximately 7, although varying somewhat with conditions (e.g., the optimum pH increases with dilution of the urea, so that it is desirable to have the final pH for quantitative decomposition in the neighborhood of 8); that a given amount of enzyme decomposes per minute a given amount of urea, *regardless of the concentration of the latter, until it has been nearly all* decomposed. Consequently most rapid completion of decomposition is obtained by using much enzyme and little urea. Van Slyke and Cullen (46) also studied the conditions for rapid aeration of ammonia from blood or urine into acid receiving solutions, and found among other things that the decrease in gas solubility caused by saturating the aerated solution with potassium carbonate greatly accelerated the evolution of ammonia. With data obtained Van Slyke and Cullen were able to reduce to a few minutes the time required for quantitative action of the enzyme in blood and urine analyses and for the aeration of the ammonia for titration, and to devise a simple method for standardizing the activity of urease preparations.

Using the above data other authors have applied micro-methods for determination of the products of reaction, so that the determination can be made in small amounts of blood. Folin and Wu (16) introduced their nesslerization technique, so that they could easily determine the urea in 0.5 cc. of blood. Gad-Andresen (21), using 0.1 cc. of blood, aerated the ammonia formed into acid and determined it gasometrically in a special apparatus by the hypobromite method. Hindmarsh and Priestly (24) have modified Gad-Andresen's method by making the ammonia determination with Folin's Nessler solution. Feinblatt (14) and Karr (26) and others, have reduced to micro-dimensions a method of Grigaut and Guérin (22) in which the urease acts on the whole blood, which is then freed from proteins by trichloroacetic acid, the ammonia being determined by direct nesslerization of the filtrate. We have found this method to yield results averaging 1 to 3 mg. of urea nitrogen per 100 cc. higher than those obtained by aeration. Other constituents of blood filtrates apparently increase the intensity of color produced. The difference is sufficient to make the direct nesslerization undesirable when the blood urea clearance (see below) is being determined, but the method is accurate enough to indicate gross urea retention.

Not only the ammonia, but also the carbon dioxide formed by the action of urease, has been used in urea determination. Partos (39) and Aszódi (8)

have determined urea in urine by treating the latter with urease in a closed flask connected with a manometer which registers the CO_2 pressure developed when the mixture is acidified. Mirkin (35) first applied the principle to blood by determining the CO_2 content in two samples, one that had been diluted with 0.5 volume of water, the other with 0.5 volume of urease solution, the difference indicating the CO_2 formed from urea. Van Slyke (43) has recently developed rapid and accurate micro methods which are carried out in the manometric blood gas apparatus described in the present volume and yield accurate results with as little as 0.2 cc. of blood.

Behre (10) has reported results indicating that blood cells when treated with concentrated crude extracts of soy beans give off more ammonia than corresponds to the urea contents determined in the Folin-Wu blood filtrate. Addis (1) obtained a similar excess of ammonia when whole blood was treated with a great excess of jack bean urease at 38° for an hour. When liver was treated in the same way as blood the amount of ammonia formed exceeded many-fold the urea that could be extracted from the liver. Addis explained these results as due to the arginase action of the liver which splits urea from arginine contained in the bean extract, this urea being then hydrolyzed to NH_3 and CO_2 by the urease present. The cells of whole blood seem to contain slight amounts of arginase, to judge from the results of Addis and Behre. The serum contains none.

These results obtained with great excesses of crude bean extract, might seem to indicate that although the urease method gives correct results when the enzyme acts on plasma or on blood filtrate, the yields are too high when it acts on whole blood. In fact, however, the urease method when applied as outlined in this volume, with only requisite amounts of enzyme and with the digestion at room temperature, is exact also for whole blood, at least of man. Van Slyke (43) obtained the same results from whole blood and from the Folin-Wu filtrate, both by the gasometric determination of the CO_2 formed and by aeration and titration of the ammonia according to the technique of Van Slyke and Cullen (45, 46).

Xanthydrol

Fosse (19) showed that when added to urea in alcoholic acetic acid solution xanthydrol forms an exceedingly insoluble precipitate.

$$2\,O\left\langle\begin{matrix}C_6H_4\\ C_6H_4\end{matrix}\right\rangle CHOH + CO(NH_2)_2 = O\left\langle\begin{matrix}C_6H_4\\ C_6H_4\end{matrix}\right\rangle CH-NH-CO-NH-CH\left\langle\begin{matrix}C_6H_4\\ C_6H_4\end{matrix}\right\rangle O + 2\,H_2O$$

Xanthydrol + Urea = Xanthydrol ureide

Two molecules of xanthydrol combine with one of urea, so that the ureide weight is exactly seven-fold that of the urea. The precipitate is accordingly well adapted to gravimetric determination of small amounts of urea. Xanthydrol combines with many substances other than urea, but apparently none of them occurs in human blood or urine (19). A gravimetric method for blood has been developed by Fosse, Robyn and François (18, 20) and a micro modification by Nicloux and Welter (37, 38). The latter could determine urea in 1 cc. of blood serum, but required a micro balance for the weighings. Auguste (9) determined the precipitate nephelometrically, Luck (30) by titration with permanganate, and Yoshimatsu (51) colorimetrically, with the aid of Folin's phenol reagent. Beattie (9a) dissolves the xanthydrol precipitate in 50 per cent sulfuric acid and determines colorimetrically the urea in the yellow solution. One cubic centimeter of Folin-Wu filtrate serves for this analysis. The xanthydrol precipitation appears to be quantitative and specific for urea under the conditions employed, but the greater convenience, especially for micro analyses, of the equally accurate urease methods, has apparently prevented its adoption in this country.

Heat decomposition

When heated to 150° to 200° in acid or alkaline solution urea decomposes, as when split by urease, into 2 molecules of ammonia and 1 of carbon dioxide.

$$CO\,(NH_2)_2 + 2\,H_2O = (NH_4)_2\,CO_3.$$

Heat decomposition of urea was apparently the first property used for its quantitative determination, and was the one relied upon for exact results until the urease and xanthydrol methods appeared. Bunsen (13) heated with barium hydroxide in bomb tubes for four hours at 220° to 300° and determined the CO_2 formed from the amount of $BaCO_3$ precipitated. This method was used for blood analyses in 1882 by von Schroeder (49) in his classic work on the locus of formation of urea from ammonia.

More recent heating methods have used acid solutions, in which 150° suffices for the decomposition, and substances other than urea are less liable to decomposition. This temperature has been obtained either by use of an autoclave (11, 12) or by hygroscopic salts which melt in their water of crystallization without boiling at this point. Of the latter, magnesium chloride was introduced by Folin (15), lithium chloride by Saint Martin (40). Benedict (12) concentrated urine to dryness with a mixture of zinc sulfate and potassium bisulfate and heated for thirty minutes at 162° in a bath. The autoclave method decomposes appreciable amounts of urinary substances

other than urea, but the salt fusion methods only negligible amounts. Benedict's fusion was the best of the heating methods. All of them were abandoned, even by their originators, when the more convenient and specific urease method was developed. Later Folin and Wu (16), Clark and Collip (13-*a*) and Leiboff and Kahn (28-*a*) used again the autoclave technique for Folin-Wu blood filtrates. The results are, as in urine, somewhat high, because of some ammonia formation from nitrogenous compounds other than urea, but Folin and Wu (16) state that the autoclave method rarely gives results exceeding the urease method by more than 1 mg. of urea nitrogen per 100 cc. of blood.

Gasometric Hypobromite Method

The gasometric hypobromite method introduced by Knop (27) and Hüfner (25) depends on measurement of the nitrogen gas formed by the reaction with alkaline hypobromite:

$$CO\,(NH_2)_2 + 8\,NaOH + 3\,Br_2 = 6\,NaBr + Na_2CO_3 + 6\,H_2O + N_2.$$

The evolution of the nitrogen is completed in 1 or 2 minutes, so that the method is very rapid, and it requires no standard solutions. The reaction, however, under the conditions usually chosen for analysis does not yield quite 100 per cent of the nitrogen indicated by the equation, although Krogh (28) found that a gas yield approaching 100 per cent could be obtained when but a small concentration of bromine was used. To more or less balance the negative error due to less than theoretical nitrogen formation, there are positive errors due to the fact that nitrogen gas is evolved by other substances than urea. Ammonia gives off about 95 per cent of its nitrogen, creatinine 14 per cent, uric acid and allantoin 50 per cent, creatine 67 per cent (41). The hypobromite method has, nevertheless, long been used by clinicians for urine analyses because of the ease with which approximate results could be obtained.

Ambard (2, 3) and his school have applied the method to the protein-free filtrate of blood. One of the writers has found that it yields on the average about 2 mg. of nitrogen per 100 cc. of blood from non-urea substances (44). This error is sufficient to make the method undesirable for blood urea determinations when the latter are to be used to estimate accurately the ratio between blood urea and urea output by the kidneys, but does not invalidate the analysis for the purpose of ascertaining whether there is a gross pathological retention in the blood.

For urea determinations in urine Krogh (28) found that good results were yielded by the hypobromite method if the urine was first filtered after being

treated with phosphotungstic acid, which precipitates ammonia and most of the other interfering substances listed above. Stehle (41) reports that after removal of the ammonia by means of permutit the amounts of the other interfering substances are so small that the hypobromite method gives results accurately checking those by the urease method. Van Slyke (45) has confirmed Stehle, but has found that the accuracy of the results is obtained by a balancing of small errors. If two minutes are allowed for the evolution of nitrogen with a properly prepared hypobromite solution, the nitrogen ordinarily evolved from non-urea urinary constituents almost exactly counterbalances a deficit of about 4 per cent in the nitrogen evolved from urea. If a longer time is allowed for the reaction, gradually increasing amounts of nitrogen from the creatinine, etc., are evolved, and the results become too high. Even with a two-minute period, urine with unusually large proportions of non-urea nitrogen will yield results a little high, and vice versa. Nevertheless the hypobromite method used under properly chosen conditions, yields results sufficiently accurate for many of the purposes to which urine urea figures are applied.

Besides measurement of the nitrogen gas, the hypobromite reaction could theoretically be measured by determination of (a) the CO_2 formed, (b) the bromine decrease, iodometrically, (c) the alkali decrease. The last mentioned has been proposed by Margosches and Rose (31a), who obtained theoretical results with pure urea oxidized a few minutes at water bath temperature.

Titration with mercuric salts

Titration with mercuric salts was introduced by Liebig (29). A complex salt is formed of approximately the composition, $2\,[Hg\,(NO_3)_2]\,[CO\,(NH_2)_2]$, which is insoluble in alkaline solution. If excess of urea is present, addition of a drop of the urea-mercuric salt solution to sodium carbonate solution produces a white precipitate of the above mercury-urea salt. If an excess of of mercury is present, however, a brown precipitate of mercuric oxide is also formed. Liebig's titration was performed by adding standard mercuric nitrate solution to urine, until a drop of the mixture tested with sodium carbonate showed by a brown coloration that excess of mercury was present. It was found, however, that the combining power of mercuric salts for the urinary nitrogeneous products is so general that the titration measured practically the total nitrogen. The mercury titration was therefore long ago abandoned. Nevertheless, it has recently been revived by Hench and Aldrich (23) who have found that the titration figure obtained in the saliva parallels the blood urea concentration sufficiently to reveal gross pathological increases in the latter.

UREASE PREPARATIONS

Preparation of permanent soluble urease powder and of urease solution. Van Slyke and Cullen (45, 46)

One part of jack bean meal is digested with five parts of water at room temperature, with occasional stirring, for an hour. The solution is cleared by centrifugation or by filtration through paper pulp. To 1 volume of this aqueous extract are added 10 volumes of acetone. The precipitate is filtered, dried *in vacuo* and pulverized. In the dry form the urease retains its strength indefinitely. Dry urease prepared by the above method can be obtained from E. R. Squibbs in New York.

The preparation is used in 10 per cent aqueous solution. To prepare the solution only enough water is added to the powder at first to form a paste, which is well mixed before the rest of the water is added. The aqueous solution will ordinarily keep a week at room temperature without losing more than 10 per cent of its activity. As ammonia and CO_2 may form, however, it is preferable to prepare a fresh solution the day it is used. Solutions of the enzyme more dilute than 10 per cent deteriorate more rapidly and hence are not used.

If the urease solution is to be used for ***gasometric blood urea determinations*** it is well to clarify the solution by centrifugation. The blanks with clarified solutions are somewhat lower and more constant.

Preparation of urease extract. Folin and Wu (16)

Folin and Wu extract the urease from jack beans with 30 per cent alcohol and free the extract from ammonia by means of permutit.

Wash 3 grams of permutit powder in a 200-cc. flask or bottle by decantation, once with 2 per cent acetic acid and twice with water. Add to the moist permutit 100 cc. of 30 per cent alcohol (35 cc. of 95 per cent alcohol mixed with 70 cc. of water). Introduce 5 grams of jack bean meal, shake for ten minutes and filter. The filtrate contains substantially the whole of the urease present in the jack bean powder. Folin and Wu state that it will keep at least a week at ordinary room temperature if not exposed to direct sunlight and remains good for three to six weeks if kept on ice. Its urease content is weaker than that of the Van Slyke-Cullen 10 per cent urease solution and to use it for the Van Slyke-Cullen methods the time of digestion must be prolonged.

Determination of activity of urease preparations: aeration procedure. Van Slyke and Cullen (45)

Urease as supplied by the manufacturer varies somewhat in activity, and it is desirable to test fresh supplies in the following way:

Reagent solution

Dibasic potassium phosphate (K_2HPO_4)	0.25 mol. or 43 grams
Diacid potassium phosphate (KH_2PO_4)	0.25 mol. or 34 grams
Urea	1.0 mol. or 60 grams
Water to make 1000 cc.	

In place of the 43 grams of K_2HPO_4 one may use 90 grams of $Na_2HPO_4 \cdot 12 H_2O$ or 35.5 grams of Na_2HPO_4. In place of the KH_2PO_4 one may use 35 grams of $NaH_2PO_4 \cdot H_2O$.

Procedure

Place 5 cc. of this solution in the tube of the aeration apparatus (fig. 74) and bring to 20°C. in a water bath. Add 1 cc. of enzyme solution, also at 20°C. and allow the mixture to stand at this temperature for exactly fifteen minutes. Add 6 or 7 grams of potassium carbonate or 6 cc. of saturated K_2CO_3 solution, and aerate the ammonia formed into 30 cc. of 0.1 N acid in the manner described below for the determination of urea in blood. The excess acid is titrated with 0.1 N alkali. The amount of 0.1 N ammonia from the urea decomposed should be, for use in the Van Slyke-Cullen method, at least 10 cc. This is equivalent to 30 mg. of urea decomposed in fifteen minutes by the 100 mg. of urease, or 1.2 mg. of urea per milligram of urease per hour.

A gasometric measure for urease activity is described on page 377.

Urea in Urine

Choice of methods

When urease is used to change the urea into ammonium carbonate, the the analyst may for technique of determination choose between three procedures: 1, aerating the ammonia into standard acid for titration; 2, absorbing the ammonia with permutit and liberating it with alkali solution for colorimetric determination by Nessler's solution; 3, gasometric determination of the CO_2 of the ammonium carbonate.

For use without urease we have described the heating procedure of Benedict, the gravimetric xanthydrol method of Fosse, and a gasometric hypobromite method, performed after removal of the preformed urinary ammonia with permutit.

Of the six methods, all are capable of better than 1 per cent accuracy except the colorimetric and hypobromite procedures, which suffer respectively the limitations of colorimetry and specificity. The colorimetry may involve errors of 2 to 3 per cent, and the error of the hypobromite method, due to varying amounts of N_2 formation from non-urea substances, may be as high as 4 per cent.

In rapidity and in simplicity of reagents the hypobromite method excels: the only preliminary treatment is shaking the urine with permutit to remove performed ammonia, and the gasometric analyses can be carried out at the rate of one every four minutes. The gasometric urease method is almost as rapid. The urease methods based on ammonia determination are longer, because they involve isolation, either by aeration or by permutit absorption, of the ammonia before it can be determined. The heating method requires still more time, and, like the other ammonia methods, demands either removal or separate determination of the preformed ammonia in the urine.

Of the methods described, the three most desirable for exact routine work, according to the authors' experience, are the gasometric urease method, the aeration urease method, and Benedict's heating method, in the order given. The gasometric urease method combines speed and accuracy as do no others at present available. It is therefore the method of choice when a manometric apparatus is available. Equally accurate, but requiring more time and many more pieces of apparatus for a series of analyses, are the urease method with aeration and, still more time consuming, the Benedict heating method.

As alternatives, the hypobromite method may be used when strict accuracy is not required, and the colorimetric urease method has proved its value when facilities for the aeration or gasometric urease procedures were not available (50). The gravimetric method, requiring no special apparatus or standard solutions, may be convenient when only an occasional urine urea analysis is required.

The two gasometric methods are described on pages 361 and 379 in chapter VII. The other four procedures are detailed below.

UREASE METHOD WITH AERATION AND TITRATION, FOR BOTH UREA AND AMMONIA. VAN SLYKE AND CULLEN (45, 47)

Reagents

Ten per cent solution of urease. See p. 545.

Phosphate buffer solution. 6 grams of acid potassium phosphate (KH_2PO_4) and 2 grams of anhydrous Na_2HPO_4 (or 5 grams of $Na_2HPO_4 \cdot 12 H_2O$) are dissolved in water and diluted to a liter.

0.02 N hydrochloric acid.

0.02 N sodium hydroxide, kept in a paraffined bottle or prepared each week by dilution from 0.1 N solution.

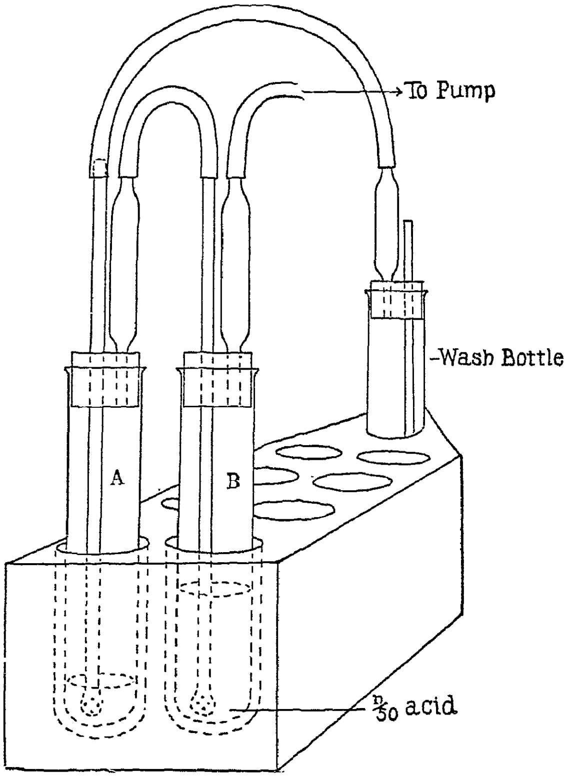


FIG. 74. Apparatus for the determination of urea by aeration of the ammonia formed by action of urease. Van Slyke and Cullen (47).

Potassium carbonate, saturated solution. 900 grams of K_2CO_3 dissolved in 1 liter of water.

Caprylic alcohol.

Alizarin red or methyl red indicator.

Apparatus

The apparatus consists of Pyrex test tubes 25 by 200 mm. arranged in pairs, as are A and B of figure 74, and a means of suction or pressure to draw

or drive air through the tubes. An extra tube containing 5 per cent sulfuric acid is inserted before the series to remove any traces of ammonia that might be present in the air.

Procedure

The urine sample taken is ordinarily 1 cc., but if the urine is unusually concentrated 0.5 cc. is taken, and if unusually dilute, 2 cc. Place the sample in tube *A* (fig. 74), together with 5 cc. of the phosphate buffer solution, 2 drops of caprylic alcohol, and, just before closing the tube, add 1 cc. of the urease solution. Put the stopper in place, mix the solutions in the tube, and let the tube stand fifteen minutes for the urease to act. While the enzyme is acting 25 cc. of 0.02 N HCl are measured into tube *B*, and the tubes are connected as shown.

After the fifteen minutes have passed run the air current for half a minute to sweep over into *B* any ammonia that has escaped from the urine into the gas space of tube *A*. Then open *A* and add either 4 to 5 grams of solid K_2CO_3 or about 10 cc. of the saturated solution. The air current is now passed, gently for the first minute, then as rapidly as may be without undue splashing or foaming. The aeration is complete in 15 minutes with a fairly rapid current (5 liters of air per minute) but requires more time with slower currents. The necessary time should be ascertained by trial with the current used, and a safe margin allowed. Several pairs of tubes connected in series can be aerated with one stream of air.

When the aeration is finished the excess acid in *B* is titrated with 0.02 N sodium hydroxide. The titration indicates ammonia + urea.

A blank analysis is performed on the reagents. The carbonate especially is likely to contain traces of ammonia.

Calculation

$$\text{Grams of urea + ammonia N per liter urine} = \frac{0.28\ (A_1 - B_1 - C)}{V}$$

A_1 is the cubic centimeters of 0.02 N acid placed in the receiving tube, B_1, is the cubic centimeter of 0.02 N alkali required to titrate the excess acid after the aeration, C is the value of $A - B$, obtained in a blank analyses of the reagents and V represents the number of cubic centimeters of urine used as sample for the analysis.

The ammonia, without the urea, is determined by simultaneous aeration of another pair of tubes. In the *A* tube are placed 5 cc. of urine and a drop of cuprylic alcohol, in the *B* tube 25 cc. of 0.02 *N* HCl.

K_2CO_3 is added to the A tube and the aeration is conducted as above directed.

$$\text{Grams of ammonia N per liter} = 0.056\ (A_2 - B_2 - C).$$

Where A_2 represents the cubic centimeter of 0.02 N acid in the determination of ammonia alone, B_2 the cubic centimeter of 0.02 N alkali used in the titration, and C is the value of $A_2 - B_2$ obtained in a blank analysis of the reagents.

Urea nitrogen is calculated by subtracting the ammonia nitrogen from the urea + ammonia nitrogen.

The percentage of urea itself can be calculated by using 0.060 and 0.012 in place of the factors 0.028 and 0.0056 in the above two formulas.

Points to be noted in determination of ammonia by aeration (47)

A slow current of air should be used during the first two minutes, as the ammonia may otherwise be driven off so rapidly at the start that a slight amount escapes absorption in the receiver. After the first two minutes one may use as rapid a current as the apparatus will stand.

In order to drive off all the ammonia in an apparatus set up exactly as described above, 75 liters of air is sufficient (25 liters drives off 98 per cent). The length of time required to complete the aeration depends on the rate at which this volume of air is drawn.

In order to assure complete absorption of ammonia with a current as rapid as 5 liters of air per minute the column of acid in the receiving tube must be at least 5 cm. high. The use of a wider receiver with less depth of acid is not permissible.

In order to drive off all the ammonia with 75 liters of air, the solution from which it is driven must contain at least 1 gram of potassium carbonate for each 2 cc. of solution.

The inlet tubes for air in A and B must reach to the bottom of the solutions.

Standard alkali solutions increase in titratable alkali on standing in contact with glass, as they dissolve more alkali from it. The effect is most marked with dilute solutions, such as 0.02 N and 0.01 N NaOH. These should be kept in paraffin-lined bottles, and portions which stand more than a day in burettes should be discarded.

Separate sets of tubes and stoppers must be employed as containers for the standard acid and for the urine-urease solutions, because after concentrated alkali carbonate has been added to the latter it is difficult to wash off with water all traces of the alkali. Enough may remain to affect the titration of the 0.01 N acid if the same tube is subsequently used for this purpose.

Tubes which are used for nesslerization can not be safely employed for urea decomposition. The adherent trace of mercury salt may inactivate the urease.

New rubber tubing before it is used for connections must be thoroughly washed with water or steam to remove the "bloom," which often contains ammonia.

Any of the reagents, especially the carbonate, may contain a trace of ammonia. It is therefore necessary, from time to time, to run blank determinations on the reagents and to redetermine the correction indicated by C in the calculation formula above. This can be done by omitting the blood and subjecting reagents, including the urease solution, to the procedure of a regular analysis.

When the aeration method is applied to *blood* an occasional sample is encountered with an unusual tendency to foam. This may evince itself only after the aeration has proceded for some time. Under these conditions the further addition of caprylic alcohol is usually ineffectual. In such a case 2 or 3 cc. of ethyl alcohol are introduced by momentarily disconnecting the rubber tubing from the inlet tube of the digestion mixture and allowing the alcohol to be drawn in through the inlet tube.

In the exceptional specimen of blood that contains more than enough urea to neutralize all the acid, additional acid added through the inlet of tube B (fig. 74) will save the analysis if the acid is introduced as soon as the indicator has changed color.

When aeration is completed the vacuum pump, if of the water variety, should be disconnected from the tubes before it is shut off, or water may suck back into the tubes. If more than four pairs of tubes are run in one series, disconnect the series in the middle before stopping the air current.

COLORIMETRIC UREASE METHOD WITH PERMUTIT. WAKEMAN AND MORRELL (50)

Reagents

Ten per cent urease solution, see page 545.

Nessler's solution, see page 532.

Standard ammonium sulfate solution containing 0.1 mg. of ammonia nitrogen per cubic centimeter, see page 533.

Procedure

Sufficient urine to contain about 2 mg. of urea nitrogen is measured into a 100-cc. Erlenmeyer flask and enough water is added from a

burette to dilute the urine to exactly 20 cc. Three grams of washed dust-free permutit powder are measured approximately by volume in a marked test tube or graduate and added to the urine. The mixture is shaken gently for five minutes to remove preformed ammonia. Of the clear supernatant solution 10 cc. are pipetted into a 250-volumetric flask. One cubic centimeter of 10 per cent urease solution is added, and the mixture is permitted to stand twenty minutes at room temperature. Then 3 grams of permutit are added and the mixture is shaken gently for five minutes to complete absorption of the newly formed ammonia. The powder is then washed by decantation with about 200 cc. of water to remove urine products other than the ammonia on the permutit. None of the powder must be lost during the washing. After the latter is finished 10 cc. of water and 1 cc. of 10 per cent sodium hydroxide are added to the permutit powder in the flask, and the mixture, after being shaken gently, is diluted with water to about 200 cc. Twenty cubic centimeters of Nessler's reagent are then added, and the whole volume is made up to 250 cc. A standard is at the same time prepared in the same manner except that an equal quantity of water is substituted for the urine and an amount of standard ammonia solution containing 1 mg. of nitrogen is added after the volume of the solution has been brought to 200 cc.

Standard and unknown are compared in the colorimeter.

Calculation

$$\frac{n\ S}{V\ U} = \text{grams of urea nitrogen per liter of urine.}$$

$$\frac{2.14\ n\ S}{V\ U} = \text{grams of urea per liter of urine.}$$

Where S and U = colorimetric readings of standard and unknown respectively; V = the volume of undiluted urine represented by the sample taken for analysis; and n the milligrams of nitrogen in the standard.

If separate ammonia determinations are made on the urine the preliminary removal of preformed ammonia in the above analysis can be omitted. The preformed ammonia is then determined with the ammonia formed from urea, and from the sum the separately determined ammonia nitrogen is subtracted.

BENEDICT'S HEATING METHOD (12)

Apparatus

Two baths each consisting of an 800-cc. Pyrex or porcelain beaker partly filled with sulfuric acid are required. One is heated to 125° to 135°, the other to 162° to 165°.

Pyrex test tubes of about 100-cc. capacity.

The apparatus for macro Kjeldahl distillation described on page 520 in the nitrogen chapter.

Reagents

Zinc sulfate.
Potassium bisulfate.
Ten per cent sodium hydroxide solution.
0.1 N acid and alkali.

Procedure

Five cubic centimeters of urine are introduced into a 100-cc. Pyrex test tube and approximately 3 grams of potassium bisulfate and 1 to 2 grams of zinc sulfate are added. A bit of paraffin and a little powdered pumice are introduced to facilitate smooth boiling, and the mixture is boiled practically to dryness, either over a free flame, or more conveniently by partial immersion in the bath at 125° to 135°. The tube is then placed in the bath at 162° to 165° (not lower) and left there for an hour. During this heating the tube must be immersed in the hot fluid for three-fourths of its length. At the end of the hour the tube is removed from the bath, the acid is washed off under the tap, a little distilled water is poured into the tube, and the contents are dissolved with the aid of heat and washed into a Kjeldahl distilling flask. A small amount of black pigment adhering to the sides of the test tube may be disregarded, as it encloses no ammonia salts. The fluid in the distillation flask is diluted to about 300 cc. and is rendered alkaline by the addition of 15 or 20 cc. of 10 per cent sodium hydroxide. Distillation is then carried on for forty minutes, as described on page 522 for macro Kjeldahl analyses.

Calculation

The calculation of urea nitrogen is the same given for Kjeldahl nitrogen in page 525. If one desires to calculate urea instead of nitrogen, the factor 30 is inserted in place of 14 in the calculation formula.

GRAVIMETRIC XANTHYDROL METHOD OF FOSSE (18, 19)

Reagents

Ten per cent solution of xanthydrol in methyl alcohol.
Methyl alcohol.
Glacial acetic acid.

Procedure

Urine, if of ordinary concentration, is diluted 10-fold with water. Of the solution 10 cc., containing 1 cc. of urine, are mixed with 35 cc. of glacial acetic acid. Five cubic centimeters of the xanthydrol solution are then added in 5 separate portions, 1 cc. every ten minutes. This manner of addition causes the precipitate of ureide to separate in crystals of a character suitable for filtration. The mixture is allowed to stand one hour, after the last cubic centimeter of xanthydrol reagent has been added, for precipitation to become complete.

To collect the crystals a porcelain funnel is used in which is a perforated plate 7 or 8 cm. in diameter. A slightly wider parchment filter paper is softened with water and fitted into this funnel, the edges of the paper being turned up about the walls of the funnel. The precipitate is collected with suction on the paper, and is washed several times with methyl alcohol. The precipitate and filter paper are then dried in a steam oven or other warm place.

The dry precipitate forms a felt-like mat which can be readily and quantitatively detached from the paper. It is picked up with a pair of pinchers and weighed directly on the balance pan.

Calculation

The weight of the precipitate divided by 7 gives the weight of urea.

$$\text{Grams of urea per liter urine} = \frac{\text{Grams of precipitate}}{7\ V} \times 1000$$

$$= \frac{143}{V} \times (\text{Grams of precipitate}).$$

$$\text{Grams of urea nitrogen per liter urine} = \frac{66.7}{V} \times (\text{Grams of precipitate}).$$

V = cubic centimeters of urine represented in sample. ($V = 1$ ordinarily.)

Urea in Blood

Choice of methods

Urease is the specific reagent *par excellence*; it can be used for urea determination without even removing the blood proteins. Furthermore it has the advantage that it acts at room temperature and quickly, in 1 minute in fact under the conditions of the gasometric determination. We have accordingly given most space to methods which utilize this enzyme. Two

urease methods, the aeration-titration procedure, and the gasometric, are applied directly to whole blood or serum, without removal of the proteins. For use on the Folin-Wu filtrate, where the dilutions are too great for accurate titration, the gasometric urease method also applies, and two colorimetric urease procedures are given, Folin and Wu's in which the ammonia formed is distilled, and Wakeman and Morrell's, in which the ammonia is separated by absorption with permutit from the urease and other filtrate constituents before nesslerization.

In *accuracy* among the above procedures the aeration-titration and gasometric are regarded by the authors as somewhat superior to the colorimetric. Both titrimetric and gasometric urease procedures are capable of precision within 1 per cent, while nesslerization and colorimetry are likely to involve errors of 2 or 3 per cent.

In *quantity of blood* the aeration-titration method is limited to conditions permitting use of 3-cc. samples. The colorimetric procedures each require only the filtrate from 0.5 cc. of blood. The gasometric can be carried out on as little as 0.2 cc. of whole blood.

Since urease may not always be available we have given three methods based on other principles: the gasometric hypobromite reaction with the blood gas apparatus, the heating and nesslerization method, and the Beattie colorimetric xanthydrol procedure. The first two require each the filtrate from 0.5 cc. of blood; the last only that from 0.1 cc. The hypobromite procedure gives results with a positive error which averages about +2 mg. of nitrogen per 100 cc., because of the reaction of the hypobromite with other substances in blood filtrate. As stated by Folin and Wu (16) the heating method gives results usually a little high, but seldom by more than 1 mg. of nitrogen per 100 cc. The writers have not tested Beattie's method, but it is simple, and the originator's data indicate that it checks the urease method within the limit of the colorimetric error, about 1 mg. of urea nitrogen per 100 cc. of blood.

For *rapidity* the premier method among those described is the gasometric hypobromite procedure carried out in Folin-Wu filtrate with the manometric apparatus; this requires but three minutes. It is, however, also the least exact method. It suffices well when the only question is whether the blood urea exceeds the maximum normal range or not. For this a constant plus error of 2 mg. urea nitrogen per 100 cc. is not prohibitive. Only slightly less rapid, however, is the exact gasometric-urease method performed on the Folin-Wu filtrate. This is the procedure now employed for several years for routine work in the authors' laboratories.

The other methods, which required heating, aeration, distillation, treat-

ment with permutit, or filtration of the xanthydrol ureide before the final measurements, are not so rapid, but all permit a large number of determinations to be performed in a half-day's work. Of these, the writers prefer the aeration-titration procedure.

The gasometric urease and hypobromite methods are described on pages 361 to 379 in chapter VII. The other methods follow below.

UREASE METHOD OF VAN SLYKE AND CULLEN (45, 47) APPLIED DIRECTLY TO WHOLE BLOOD OR PLASMA, WITH AERATION AND TITRATION OF THE AMMONIA FORMED

The same *apparatus* employed for the aeration-urease method with urine (fig. 74) is used for blood. The same *reagents* are also used, except that for blood 0.01 N instead of 0.02 N alkali and acid are used in the titration.

Procedure

The procedure is the same as for urine (p. 547) except in the following details.

The sample of blood is 3 cc., and it is mixed in tube *A* (fig. 74) with 3 cc. of the phosphate solution, 5 drops of caprylic alcohol, and 1 cc. of urease solution. Only five minutes need be allowed for completion of the reaction, although fifteen minutes standing with the urease does no harm. Unnecessarily prolonged contact between blood and enzyme is to be avoided, because of the possibility of slow formation of ammonia by action of arginase in the blood cells on arginine in the enzyme preparation. (1, 10.)

The amount of 0.01 N HCl measured into tube *B* (fig. 74) is 15 cc. Only very rare bloods from uremic subjects require more.

The rest of the determination is carried out exactly as the urine analysis.

Calculation

$$10\ (A - B - C) = \text{milligrams of urea per 100 cc. of blood.}$$

$$4.66\ (A - B - C) = \text{milligrams of urea nitrogen per 100 cc. of blood.}$$

$$0.0466\ (A - B - C) = \text{grams of urea nitrogen per liter of blood.}$$

A signifies the cubic centimeters of 0.01 N acid, usually 15, placed in the receiving tube, *B*, the cubic centimeters of 0.01 N alkali used in the titration, and *C* the value of $A - B$ found in a blank determination on the reagents.

The "Points to be noted in determination of ammonia by aeration" on page 550 are to be observed.

UREASE METHOD OF FOLIN AND WU (16) APPLIED TO BLOOD FILTRATE, WITH AERATION OR DISTILLATION, AND NESSLERIZATION

Reagents

Urease solution, see page 545.

Nessler's solution, see page 532.

Phosphate buffer solution, 0.5 molecular Na_2HPO_4, prepared as described on page 368.

Standard ammonia solution, 0.1 mg. ammonia N per cubic centimeter, see page 533.

Procedure

Five cubic centimeters of tungstic acid blood filtrate (see p. 65) are placed in tube *A* (fig. 74), as above described, with 0.2 cc. of 10 per cent Van Slyke-Cullen jack bean urease solution or 1 cc. of Folin-Wu jack bean extract and 0.15 cc. of phosphate buffer solution. The digestion and aeration are carried out as described above. The receiving tube B, however, is marked in this case at the point indicating 25 cc. At the end of the aeration the 15 cc. of acid receiving solution are diluted to 20 cc. and 2.5 cc. of Nessler's solution are added. The tube is filled to the 25 cc. mark with water, and the solution is compared in a colorimeter with a standard prepared by diluting ammonium chloride or sulfate solution containing 0.3 mg. of nitrogen to 80 cc., adding 10 cc. of Nessler's solution and making up to 100 cc. with water. "Octyl alcohol should not be used: it causes turbidity in the Nessler's solution."

If preferred the ammonia may be distilled instead of aerated into acid. To prevent bumping two glass beads or clay pebbles or, most efficient of all, a small piece of broken porous clay plate, is added. The solution is rendered alkaline with 2 cc. of saturated borax solution and distilled as described for micro-Kjeldahl determinations.

Calculation

The standard in the colorimeter should be set at 20 mm.

$$\frac{0.15\,S}{U} = \text{grams of urea nitrogen per liter of blood.}$$

$$\frac{15\,S}{U} = \text{milligrams of urea nitrogen per 100 cc. blood.}$$

$$\frac{32.2\,S}{U} = \text{milligrams of urea per 100 cc. blood.}$$

where *S* and *U* are the readings of standard and unknown respectively.

UREASE DECOMPOSITION OF WHOLE BLOOD FOLLOWED BY NESSLERIZATION OF SOMOGYI BLOOD FILTRATE. VAN SLYKE AND PLAZIN (UNPUBLISHED)

The whole blood or plasma is treated with urease. The proteins are then precipitated by Somogyi's zinc method, and the filtrate is nesslerized.

As mentioned on page 540, numerous colorimetric blood urea methods have been proposed in which urease is permitted to act on the blood, the proteins are then precipitated, and the blood filtrate is nesslerized. With the protein precipitants previously used, such methods give somewhat high results, because the blood filtrates contain substances, other than urea or ammonia, which give a significant color with Nessler's reagent. When Somogyi's zinc method (page 481) is used to remove the blood proteins, however, a filtrate is obtained which remains quite colorless when Nessler's reagent is added. When the blood is previously treated with urease the ammonia nitrogen found in the filtrate by nesslerization equals, within the limits of colorimetric determination, the urea nitrogen found by titration or gasometric urease methods.

Reagents

Ten per cent urease solution (see page 545).

Zinc sulfate and sodium hydroxide for the Somogyi precipitation (see page 481).

Nessler's solution (see page 532).

Standard ammonium sulfate solution containing 0.015 mg. of ammonia nitrogen per cubic centimeter. Fifteen cubic centimeters of the standard solution described on page 533 are diluted to 100 cc. with ammonia-free water.

Procedure

The blood sample is mixed with one-tenth its volume of 10 per cent urease solution, and is permitted to stand thirty minutes at room temperature. The proteins are then precipitated by Somogyi's method, as described on page 481, to obtain a 1:10 filtrate.

To 5 or to 10 cc. of the standard, according to the volume required for the colorimeter, add 0.5 or 1 cc., respectively, of Nessler's solution. Then similarly nesslerize 5 or 10 cc. of the blood filtrate. Compare the two solutions at once in a colorimeter. If the nesslerized blood filtrate stands, it is likely in a few minutes to develop a turbidity.

If the blood urea is high, so that the nesslerized filtrate is much darker than the standard, the reading is nevertheless taken in the colorimeter. From this reading one calculates approximately how

many times more concentrated the ammonia is in the filtrate than in the standard. Another portion of the filtrate is then diluted 2, 3, 5, or 10-fold, to bring it to approximately the ammonia content of the standard. The diluted filtrate is then nesslerized and compared with the standard.

Calculation

$$\frac{0.15\ S}{DU} = \text{grams of urea nitrogen per liter of blood.}$$

$$\frac{15\ S}{DU} = \text{milligrams of urea nitrogen per 100 cc. of blood.}$$

$$\frac{32.2\ S}{DU} = \text{milligrams of urea per 100 cc. of blood.}$$

S and U represent readings of standard and unknown respectively. D is the number of times the blood filtrate is diluted, in analyses where high blood urea necessitates dilution.

ACID-HEAT DECOMPOSITION AND NESSLERIZATION OF FOLIN-WU FILTRATE. FOLIN AND WU (16); LEIBOFF AND KAHN (28A)

In both these methods the procedure introduced by Benedict and Gephart (11) is used, of heating the acidified urea solution to 150° for ten minutes to hydrolyze the urea to ammonia. Folin and Wu do the superheating in an autoclave, while Leiboff and Kahn have devised special pressure tubes of glass which make an autoclave unnecessary. Folin and Wu ((16), p. 91) state that they abandoned attempts at direct nesslerization of the hydrolyzed blood filtrate, because "very small amounts of ammonia cannot be nesslerized in the presence of either amino acids or peptones." They consequently boiled off the ammonia and nesslerized the distillate. Leiboff and Kahn, however, disregard interfering substances and nesslerize directly in the hydrolyzed blood filtrate. They present figures showing by this procedure results usually higher than by the urease method, performed with distillation; but the difference seldom was as great as 1 mg. of urea nitrogen per 100 cc. of blood. It is possible that their apparent immunity from the trouble experienced by Folin and Wu in attempting direct nesslerization of the hydrolyzed blood filtrate is due to the use by Leiboff and Kahn of the modified Nessler reagent of Koch and McMeekin, which these authors have claimed to be somewhat less liable than the original reagent used by Folin and Wu to interference from non-ammonia substances. The difference, if any,

between results obtained by nesselerization of the hydrolyzed blood filtrate and nesslerization of the distillate is a point which the writers have not had opportunity to determine.

Autoclave hydrolysis. Folin and Wu (16)

Five cubic centimeters of Folin-Wu filtrate (p. 65) are placed in a 75-cc. test tube of Pyrex glass. One cubic centimeter of 1 N sulfuric acid is added. The mouth of the tube is covered with tin foil, and the tube is heated in an autoclave at 150° (inside autoclave temperature) for ten minutes.

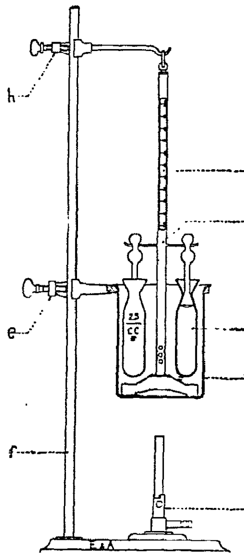


FIG. 75. Apparatus of Leiboff and Kahn (28a) for hydrolysis of urea by heating to 150° in pressure tubes. The heating is done in an oil bath, *d*. This bath consists of a metal container capable of holding six tubes, thus allowing simultaneous determinations. In the container is placed a removable metal rack, the center of which holds a 200° thermometer inclosed in a metal jacket to prevent breakage. To the upper part of the thermometer jacket is attached a circular disc, *b;* with six grooves into which the external ends of the stoppers slip, thus holding the pressure tubes suspended in the oil. The edges around the grooves are slightly turned upwards in order to prevent the tubes from falling off. This apparatus can be obtained from Eimer and Amend, New York.

Pressure tube hydrolysis. Leiboff and Kahn (28a)

The same mixture is placed in one of the pressure tubes shown in figure 75. Close the tube by pulling the stopper upwards into position and turning it slightly to make it fit snugly. Place the rack holding the

tube in the oil bath in such a way that the surface of the solution in the tube is somewhat below the level of the surface of the oil.

With a Bunsen burner raise the temperature of the oil to 150° and keep it there for ten minutes. A variation of a few degrees is not important.

Any oil with a flash point sufficiently above 150° serves for the bath. Leiboff and Kahn recommend the petroleum oil preparation sold as "Nujol."

Distillation of the ammonia

The ammonia my be distilled, either from the hydrolysis tube or from another tube or micro Kjeldahl flask, by either of the techniques shown for micro Kjeldahl determinations in the chapter on nitrogen (pp. 529 and 531). In this case, however, instead of adding concentrated NaOH as the alkali to set the ammonia free, ***saturated borax solution*** is added. Sodium hydroxide would split off ammonia from non-urea nitrogenous constitutents in blood. Borax is sufficiently alkaline to free ammonia quantitatively, but is not alkaline enough to decompose other nitrogenous substances present.

The distillate is caught in a test tube or flask which contains 2 cc. of 0.1 N acid and is marked to contain 25 cc. of fluid.

Nesslerization

In this analysis the amount of urea in normal blood yields only enough ammonia to neutralize 0.5 to 1.0 cc. of N/70 acid. Collip and Clark (13a) prefer even with such small amounts to titrate the ammonia, which they distil with stream as described on page 531. Most workers would probable prefer to nesslerize such small amounts.

Either in the distillate or in the tube where the hydrolysis has occurred the solution is diluted to approximately 20 cc. with water, and 3 cc. of Koch and McMeekin's Nessler solution (p. 532) are added. Water is then added to the 25-cc. mark, and the solution is mixed and prepared with a standard solution in a colorimeter.

Calculation

The preparation of the standard and the calculation are the same as described above for Wakeman and Morrell's method. (The sample of Folin-Wu filtrate there used is twice as great as here, but it is also there diluted twice as much for nesslerization, so that the same calculation formula serves in the present method.)

XANTHYDROL COLORIMETRIC METHOD OF BEATTIE (9A)

The method is based on the gravimetric method of Fosse, Robyn and François (20) in which urea is precipitated as dixanthylurea from deproteinised blood-serum by the addition of an alcoholic solution of xanthydrol. Fosse's method has beeen carried out on a micro-sale by Nicloux and Welter (37), and was found to give reliable results for quantities of urea as small as 0.05 mg.

In the following method the dixanthylurea precipitate is prepared according to Nicloux and Welter, but, instead of being weighed, it is dissolved in sulfuric acid (50 per cent by volume) in which xanthydrol and its compounds dissolve, producing an intense yellow color. This color reaction is of an intensity which makes it possible to detect differences of 0.14 mg. of dixanthylurea, equivalent in the analysis to 1 mg. of urea nitrogen per 100 cc. of blood.

Reagents

Xanthydrol. Five per cent solution in methyl alcohol.

Glacial acetic acid.

Sulfuric acid. Fifty per cent by volume. One volume of concentrated sulfuric acid added to 1 volume of water.

Standard dixanthylurea solution.

1. Dilute 4 cc. of a 0.01 per cent urea solution to 10 cc. with water. Treat 1 cc. of this solution in the same way described below for the treatment of 1 cc. of Folin-Wu blood filtrate, obtaining 10 cc. of a solution in 50 per cent sulfuric acid which contains 0.04 mg. of urea. This standard serves if one desires to calculate the results in terms of milligrams of urea per 100 cc. of blood.

2. If the results are to be calculated in terms of urea nitrogen, one measures from a burette into a 10 cc. flask 4.29 cc. of the 0.01 per cent urea solution, dilutes to the mark, and prepares the standard with 1 cc. of this solution. The resultant 10 cc. of dixanthylurea contains 0.02 mg. of urea nitrogen.

Procedure

0.5 to 1.0 cc. of blood is deproteinised by the method of Folin-Wu (p. 65).

Place 1 cc. of the filtrate (= 0.1 cc. blood) in a centrifuge tube.

Add 1 cc. of glacial acetic acid, and 0.2 cc. of a solution of 5 per cent xanthydrol in methyl alcohol.

Allow this to stand for five minutes. Then stir it with a glass rod,

and allow it to settle again for half to one hour, or centrifuge for twenty to thirty minutes.

(When the quantity of urea is small, 20 mg. per 100 cc. of blood or less, the longer time is necessary to ensure complete precipitation.)

Filter off the precipitate by suction through a small Gooch crucible packed with asbestos.

Wash alternately three times with 2 cc. portions of methyl alcohol and distilled water, preferably saturated with dixanthylurea.

During the washing with methyl alcohol detach the pump until the methyl alcohol has almost drained through, to allow complete solution of the excess xanthydrol.

Test the third methyl alcohol washing with 50 per cent sulfuric acid. If no yellow color appears, the washing is complete.

Wash out the flask and place a small receiving tube inside to catch the succeeding filtrate. Pour about 5 cc. of 50 per cent sulfuric acid into the crucible.

When the precipitate is dissolved, attach the pump, and draw the yellow solution into the receiving tube. Wash with a further 4 cc. of sulfuric acid. Remove the tube and make up to 10 cc. in a graduated test tube. Compare in a colorimeter with a standard solution.

The precipitate can be filtered, washed, and dissolved in sulfuric acid in about ten minutes.

In case the unknown solution is much stronger than the standard, the unknown can be diluted with measured volumes of 50 per cent sulfuric acid.

Calculation

If the reading has been made with standard 1 in order to obtain the result in terms of urea, the calculation is:

$$\text{Milligrams of urea per 100 cc. blood} = 40 \times \frac{S}{U}.$$

If the reading is obtained with standard 2 in order to obtain the result in terms of urea nitrogen, the calculation is:

$$\text{Milligrams of urea nitrogen per 100 cc. blood} = 20 \times \frac{S}{U}.$$

S is the reading of the standard and U of the unknown in the colorimeter. If the unknown has been diluted to more than 10 cc. the results obtained by the above formulas are multiplied by $V/10$, where V is the volume in cubic centimeters to which the unknown is diluted.

THE BLOOD UREA CLEARANCE (34, 36)

For a discussion of the significance of the standard and maximum urea clearances as a measure of renal function, the reader is referred to the urea chapter of Volume I.

The following procedure for the blood urea clearance is taken from the description of Möller, McIntosh and Van Slyke (36).

The necessary data are the concentrations of urea in blood and urine and the volume of urine excreted in a measured time. The manner in which these three values are secured may be varied to suit conditions. As a routine procedure, however, the following are satisfactory:

The subject is not submitted to any previous routine, except that vigorous exercise is avoided and the previous meal should be a moderate one, preferably without coffee, which may increase the blood urea clearance. The most desirable time of day, when excretion is least liable to fluctuations, is found according to MacKay (31) in the hours between breakfast and lunch. It is well to give a glass of water before beginning the test. The subject remains quiet while the urine is collected during two succeeding periods of about one hour each. The length of the period, over or under one hour, is not essential, so long as it is exactly measured and the urine output per minute can be calculated. The chief source of error is probably the possibility of incomplete emptying of the bladder, either at the beginning or end of a period. The collection of two urine specimens affords a check on this factor. A few minutes before the end of the first hour a blood sample is drawn. Its urea content is used for calculation of the clearances during both periods. This usage is permissible, because under the conditions of the test the blood urea does not change greatly during an hour.[1]

If the urine exceeds 2 cc. per minute, as observed in an adult, or as corrected for body size (see below) in a child, the *maximum clearance* is calculated

$$C_m = \frac{U\,V}{B},$$

where U = urea concentration in urine, B = urea concentration in blood and V = urine volume in cubic centimeters per minute. It does not matter in what terms the urea concentrations U and B are expressed, so long as the same unit is used for both blood and urine, e.g., if blood urea is expressed in mg. per 100 cc., urine urea must, for this calculation, be also expressed in

[1] In the appendix is described a simplified method for determining the clearance by colorimetric comparison of blood against urine.

mg. per 100 cc. V must always be expressed in terms of cubic centimeters of urine per minute.

If the urine volume thus observed or corrected is less than 2 cc. per minute, the standard clearance is calculated.

$$C_s \quad U\sqrt{V}$$

It is advantageous as a rule to calculate both clearances in percentages of the mean normal C_s and C_m. Urea excretions observed with ordinary urine volumes and calculated in terms of C_s are thus rendered directly comparable with excretions observed with large urine volumes and hence calculated in terms of C_m. Furthermore, the percentage values thus calculated express directly percentages of average normal renal efficiency.

The percentage of average normal C_m is obtained by dividing the absolute C_m value by the mean normal C_m, 75, and multiplying by 100. Similarly the percentage of average normal C_s is obtained by dividing the absolute C_s by 54 and multiplying by 100.

$$\text{Per cent of average normal } C_m = \frac{100\ U\ V}{75\ B} = 1.33 \quad U\ V$$

$$\text{Per cent of average normal } C_s = \frac{100\ U\ \sqrt{V}}{54\ B} = 1.85 \quad U\sqrt{V}$$

Graphic calculation of C_m and C_s by charts of figure 76 and figure 77. Both the absolute and percentage C_m and C_s values are mostly readily calculated graphically by means of a slide rule or by means of the line charts in figure 76 and figure 77 respectively. When the charts are used it is necessary to calculate by arithmetic only the value of the quotient $\frac{U}{V}$, V being estimated in cubic centimeters of urine per minute, corrected for body size as outlined below if the subject is a child. A thread stretched taut across figure 76 or figure 77 intersecting the observed values of $\frac{U}{B}$ and V on the outer scales then crosses the inner scale at a point indicating both the absolute clearance and the percentage of normal.

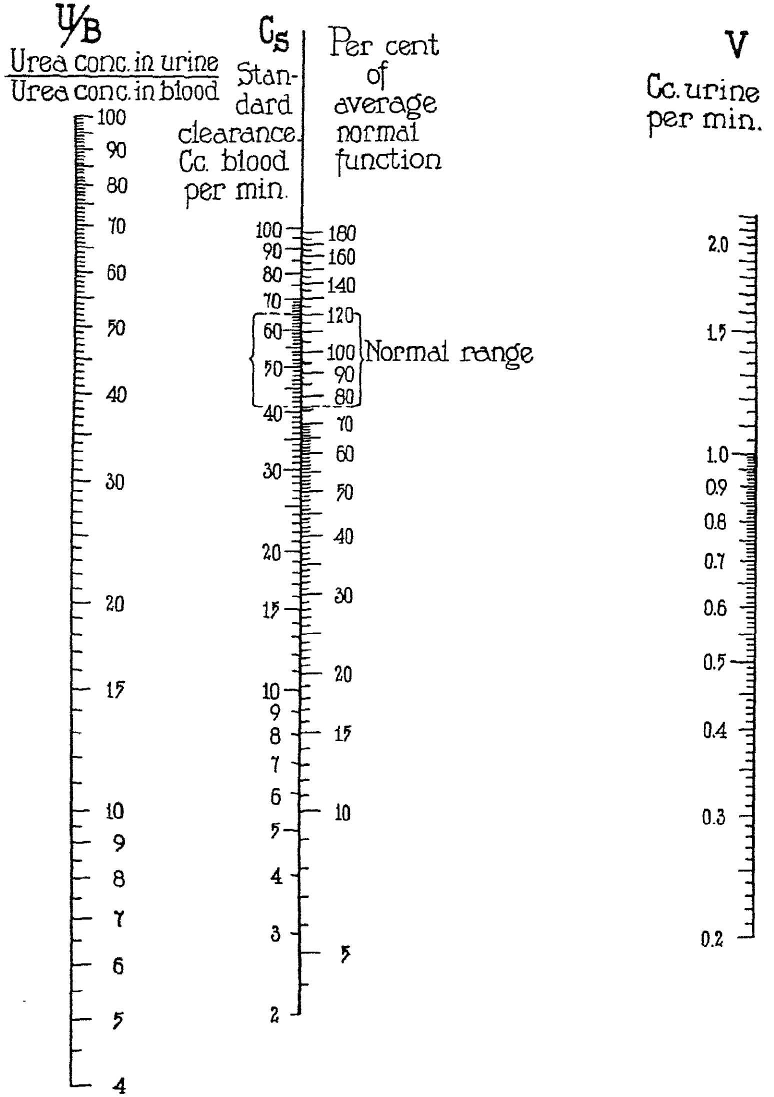


FIG. 76. Line chart for calculation of standard blood urea clearance values. Connect observed U/B and V values by a straight line. Where the line cuts the inner scale read C_s value or per cent of average normal renal function. For subjects differing markedly from usual adult size, a correction is introduced by multiplying the observed V by the factor $\frac{1.73}{\text{Square meters surface area}}$ and using the V value thus corrected for the calculation of C_s.

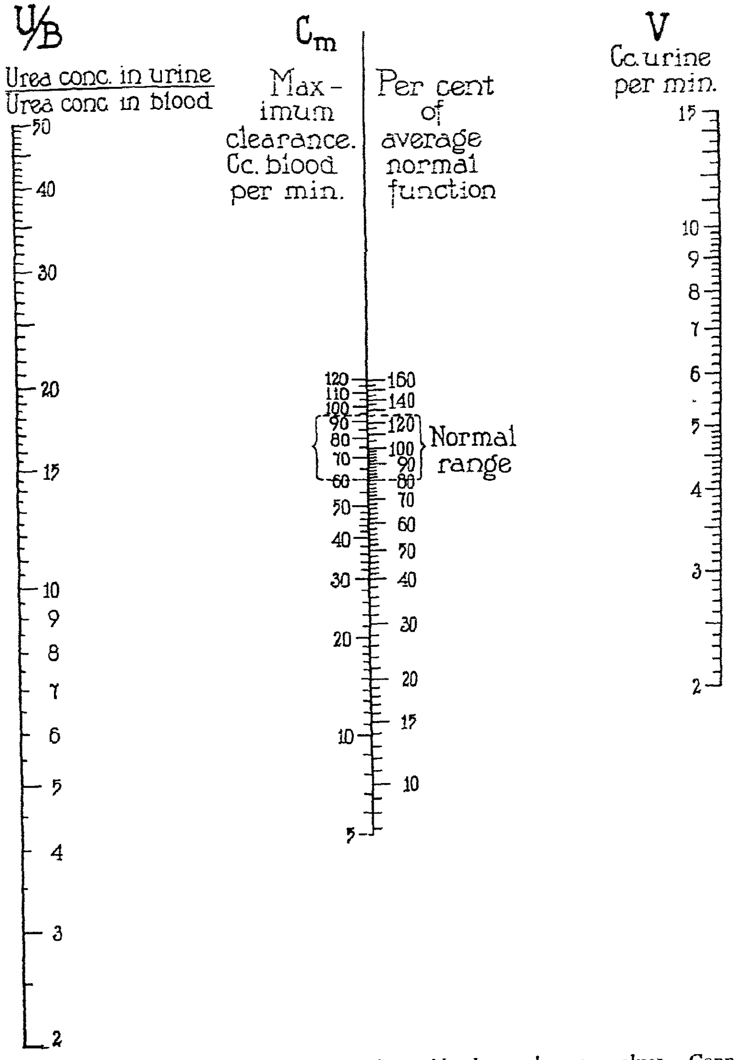


FIG. 77. Line chart for calculation of maximum blood urea clearance values. Connect observed U/B and V values by a straight line. Where the line cuts the inner scale read C_m value or per cent of average normal renal function. *For subjects differing markedly from usual adult size a correction is introduced by multiplying the observed V value by the factor* $\frac{1.73}{\text{Square meters surface area}}$ and using the V value thus corrected for the calculation of C_m.

Arithmetical calculation of C_s. For arithmetical calculation of the standard clearance the following values of the square root of V are given covering the range below the augmentation limit:

V	$\sqrt{V}$	V	$\sqrt{V}$
cc. per minute		*cc. per minute*	
0.2	0.45	1.2	1.10
0.3	0.55	1.3	1.14
0.4	0.63	1.4	1.18
0.5	0.71	1.5	1.23
0.6	0.78	1.6	1.27
0.7	0.84	1.7	1.30
0.8	0.89	1.8	1.38
0.9	0.95	1.9	1.38
1.0	1.00	2.0	1.42
1.1	1.05	2.1	1.45

Example of calculation of a normal maximum clearance

Blood urea N = 15.6 mgm. per 100 cc. = B

Urine urea N = 321.0 mgm. per 100 cc. = U

Urine volume = 210 cc. per hour = 3.5 cc. per minute = V

$$C_m = \frac{U\,V}{B} \quad \frac{321 \times 3.5}{15.6} \quad 72 \text{ cc. of blood cleared of urea per minute.}$$

Per cent of average normal function = 1.33 × 72 = 96 per cent.

Example of calculation of a normal standard clearance

Blood urea N = 14.7 mgm. per 100 cc. = B

Urine urea N = 750 mgm. per 100 cc. = U

Urine volume = 50 cc. per hour = 0.83 cc. per minute = V

$$C_s \quad \frac{U\sqrt{V}}{B} \quad \frac{750 \times 0.91}{14.7} = 46 \text{ cc. of blood cleared of urea per minute.}$$

Per cent of average normal function = 1.85 × 46 = 85 per cent.

Correction for body size is required in the case of children. McIntosh, Möller and Van Slyke (34) found that such correction was best made by multiplying the value of V in either the C_s or the C_m calculation by the factor

$$\frac{1.73}{\text{body area in sq. meters}},$$

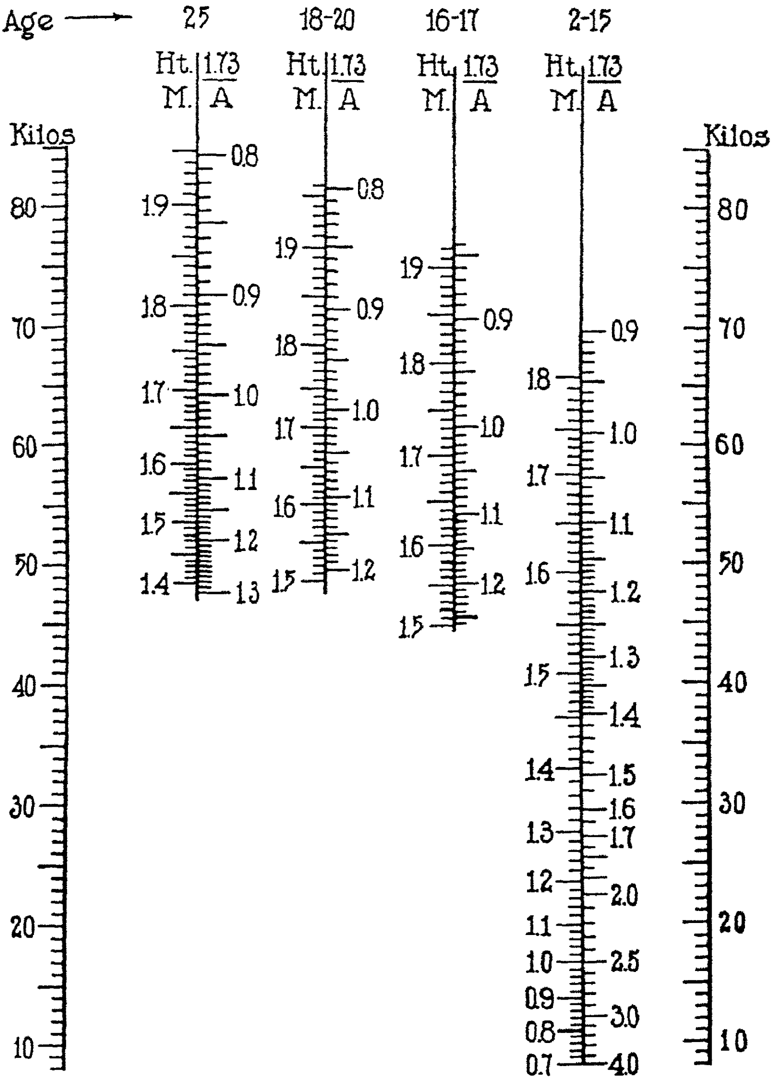


FIG. 78. Line chart for calculating correction for body size, and for estimating weight ideal for subject's height and age. The value of $\frac{1.73}{A}$ is read off opposite the height of the subject in meters, on the scale for subjects of his age, or of 25 if he is mature. A horizontal line from the same point to the weight scale on either side cuts the latter at a point indicating the ideal weight of the subject. The ideal weight is not used in calculation of the clearance corrections, but we have added the "ideal weight" scales to figure 78 because we found them of convenience for comparison with the observed weights of edematous, obese or emaciated patients.

1.73 being the mean body area for adults. By means of figure 78 the correction factor $\frac{1.73}{A}$ is determined graphically from the height of the subject. The observed value of V in cubic centimeters per minute is then multiplied by $\frac{1.73}{A}$. With the corrected value of V so obtained one proceeds with the calculation of C_m or C_s according to one of the procedures outlined above.

BIBLIOGRAPHY

1. Addis, T.: An error in the urease method for the determination of urea. *Proc. Soc. Exp. Biol. Med.*, 1928, **25**, 365.
2. Ambard, L.: Physiologie normale et pathologique des reins. Paris, 1914.
3. Ambard, L.: Remarques sur le dosage del'urée par l'hypobromite de soude. *Bull. Soc. Chim. Biol.*, 1920, **2**, 205.
4. Annett, H. E.: The urease content of certain Indian seeds. *Biochem. J.*, 1914, **8**, 449.
5. Armstrong, E. F., and Armstrong, H. E.: Studies on the processes operative in solutions. XXX. On enzyme action. XX. The nature of enzymes and of their action as hydrolytic agents. *Proc. Roy. Soc., London*, 1913, [B] **86**, 561.
6. Armstrong, H. E.; Benjamin, M. S., and Horton, E.: Studies on enzyme action. XIX. Urease: a selective enzyme. II. Observations on accelerative and inhibitive agents. *Proc. Roy. Soc., London*, 1913, [B] **86**, 328.
7. Armstrong, H. E., and Horton, E.: Studies on enzyme action. XV. Urease: a selective enzyme. *Proc. Roy. Soc., London*, 1912, [B] **85**, 109.
8. Aszódi, Z.: Beitrag zur Bestimmung des durch Urease zersetzten Harnstoffes aus der CO_2-Komponente des Umwandlungsproduktes. *Biochem. Z.*, 1922, **128**, 391.
9. Auguste, Ch.: Microdosage néphélémétrique de l'urée dans les liquides biologiques. *Compt. rend. soc. biol.*, 1923, **89**, 991.

9a. Beattie, Florence: Micro-method for the colorimetric determination of urea in blood. *Biochem. J.*, 1928, **22**, 711.

10. Behre, J. A.: Observations on the determination of blood urea. *J. Biol. Chem.*, 1923, **56**, 395.
11. Benedict, S. R., and Gephart, F.: The estimation of urea in urine. *J. Am. Chem. Soc.*, 1908, 30, 1760.
12. Benedict, S. R.: The estimation of urea. *J. Biol. Chem.*, 1910, **8**, 405.
13. Bunsen, R.: Ueber quantitative Bestimmung des Harnstoffs. *Ann. der Chem.*, 1848, **65–66**, 375.

13a. Clark, E. P., and Collip, J. B.: A procedure for the determination of urea in Folin-Wu blood filtrates by the autoclave method. *J. Biol. Chem.*, 1926, **67**, 621.

14. Feinblatt, H. M.: Determination of urea nitrogen in blood without aeration. *J. Lab. Clin. Med.*, 1923, **9**, 66.
15. Folin, O.: On the determination of urea in urine. *J. Biol. Chem.*, 1912, **11**, 507.
16. Folin, O., and Wu, H.: A system of blood analysis. *J. Biol. Chem.*, 1919, **38**, 81.
17. Folin, O., and Youngburg, G. E.: Note on the determination of urea in urine by direct nesslerization. *J. Biol. Chem.*, 1919, **38**, 111.

18. FOSSE, R.: Chimie analytique et biologique. Analyse quantitative gravimétrique de de l'urée dans l'urine. *Compt. rend Acad. Sci.*, 1914, **158**, 1588.

19. FOSSE, R.: Origine et distribution de l'urée dans la nature. Application de nouvelles méthodes d'analyse de l'urée basées sur l'emploi du xanthydrol. *Ann. Chim.*, 1916, **6**, 13.

20. FOSSE, R.; ROBYN, A., AND FRANÇOIS, F.: Analyse quantitative gravimétrique de l'urée dans le sang. *Compt. rend. Acad. Sci.*, 1914, **159**, 367.

21. GAD-ANDRESEN, K. L.: A micro urease method for the estimation of urea in blood, secretions and tissues. *J. Biol. Chem.*, 1922, **51**, 373.

22. GRIGAUT, A., AND GUÉRIN, FR.: Procédés précis de dosage de l'urée dans de faibles quantités de sang. *Compt. rend. soc. biol.*, 1919, **82**, 25.

23. HENCH, P. S., AND ALDRICH, M.: The concentration of urea in saliva. *J. Am. Med. Ass.*, 1922, **79**, 1409.

A salivary index to renal function. *Ibid.*, 1923, **81**, 1997.

Apparatus for determination of the salivary urea index. *Ibid.*, **82**, 1194.

24. HINDMARSH, E. M., AND PRIESTLY, H.: A method for the estimation of urea in 0.1 cc. of blood. *Biochem. J.*, 1924, **18**, 252.

25. HÜFNER, G.: Ueber die Anwendung des unterbromigsauren Natrons als Reagens; Bestimmung des Harnstoffs. *J. Prakt. Chem.*, 1871, **3**, 1.

26. KARR, W. G.: A method for the determination of blood urea nitrogen. *J. Lab. Clin. Med.*, 1924, **9**, 329.

27. KNOP, W.: Methode zur Bestimmung des Stickstoffs in Ammoniak-und Harnstoff-verbindungen. *Z. anal. Chem.*, 1870, **9**, 225.

28. KROGH, M.: Die Harnstoffbestimmung im Harm mit Natriumhypobromite. *Z. physiol. Chem.*, 1913, **94**, 379.

29a. LEIBOFF, S. L. AND KAHN, B. S.: A rapid and accurate method for the determination of urea in blood. *J. Biol. Chem.*, 1929, **83**, 347.

29. LIEBIG, J.: Ueber einige Harnstoff verbindungen und neue Methode zur Bestimmung von Kochsalz und Harnstoff im Harn. *Ann. der Chem.*, 1853, **85–86**, 289.

30. LUCK, J. M.: The oxidation of dixanthydryl urea, a micro method for determining urea. *J. Biol. Chem.*, 1928, **79**, 211.

31. MACKAY, E. M.: Studies of urea excretion. V. The diurnal variation of urea excretion in normal individuals and patients with Bright's disease. *J. Clin. Invest.*, 1928, **6**, 505.

31a. MARGOSCHES, B. M., AND ROSE, H.: Zur Methodik der Harnstoffbestimmung auf Grund der Hypobromitreaktion. *Biochem. Z.*, 1923, **136**, 119.

32. MARSHALL, E. K., JR.: A rapid clinical method for the estimation of urea in urine. *J. Biol. Chem.*, 1913, **14**, 283.

A new method for the determination of urea in blood. *J. Biol. Chem.*, 1913, **15**, 487.

On soy bean urease; the effect of dilution, acids, alkalies and ethyl alcohol. *J. Biol. Chem.*, 1914, **17**, 351.

33. MATEER, J. G., AND MARSHALL, E. K., JR.: The urease content of certain beans, with special reference to the jack bean. *J. Biol. Chem.*, 1916, **25**, 297.

34. MCINTOSH, J. F.; MÖLLER, E., AND VAN SLYKE, D. D.: Studies of urea excretion. III. The influence of body size on urea output. *J. Clin. Invest.*, 1928, **6**, 467.

35. MIRKIN, A.: A gasometric method for determination of urea nitrogen in the blood. Preliminary communication. *J. Lab. Clin. Med.*, 1922, **8**, 50.

36. Möller, E.; McIntosh, J. F., and Van Slyke, D. D.: Studies of urea excretion. II. Relationship between urine volume and the rate of urea excretion by normal adults. *J. Clin. Invest.*, 1928, **6**, 427.

37. Nicloux, M., and Welter, G.: Micro-analyse quantitative gravimétrique de l'urée. Application au dosage de l'urée de 1 cm. de sang. *Compt. rend. Acad. Sci.*, 1921, **173**, 1490.

38. Nicloux, W., and Welter, G.: Micro-dosage de l'urée dans le sérum sanguin normal et pathologique. *Compt. rend. soc. biol.*, 1922, **86**, 161.

39. Partos, S.: Bestimmung des durch Urease zersetzten Harnstoffs aus der CO_2-Komponent des Zersetzungsproduktes. *Biochem., Z.*, 1920, **103**, 292.

40. Saint-Martin, L. G. de.: Modification du procédé de Folin pour le dosage de l'urée dans l'urine. *Compt. rend. soc. biol.*, 1905, **58**, 89.

41. Stehle, R. L.: Note on the gasometric determination of nitrogen. *J. Biol. Chem.*, 1921, **47**, 11.
The gasometric determination of urea in urine. *J. Biol. Chem.*, 1921, **47**, 13.
Note on the gasometric determination of urea. *J. Biol. Chem.*, 1922, **51**, 89.

42. Takeuchi, T.: Urease in dem Schimmelpilze Aspergillus niger. *J. Coll. Agric. Imp. Univ. Tokyo*, 1909, **1**, 1414.

43. Van Slyke, D. D.: Determination of urea by gasometric measurement of the carbon dioxide formed by the action of urease. *J. Biol. Chem.*, 1927, **73**, 695.

44. Van Slyke, D. D.: The manometric determination of urea in blood and urine by the hypobromite reaction. *J. Biol. Chem.*, 1929, **83**, 449.

45. Van Slyke, D. D., and Cullen, G. E.: A permanent preparation of urease, and its use in the determination of urea. *J. Biol. Chem.*, 1914, **19**, 211.

46. Van Slyke, D. D., and Cullen, G. E.: The mode of action of urease and of enzymes in general. *J. Biol. Chem.*, 1914, **19**, 141.

47. Van Slyke, D. D., and Cullen, G. E.: The determination of urea by the urease method. *J. Biol. Chem.*, 1916, **24**, 117.

48. Van Slyke, D. D., and Zacharias, G.: The effect of hydrogen ion concentration and of inhibiting substances on urease. Further study on the mode of enzyme action. *J. Biol. Chem.*, 1914, **19**, 181.

49. von Schröder, W.: Über die Bildungstätte des Harnstoffs. *Arch. Exp. Path. Pharm.*, 1882, **15**, 364.
Die Bildung des Harnstoffs in der Leber. *Ibid.*, 1885, **19**, 373.

50. Wakeman, A. M., and Morrell, C. A.: Studies of the chemistry in metabolism in experimental yellow fever in Macacus Rhesus monkeys. I. The concentration of non-protein nitrogenous constituents of the blood. *Arch. Int. Med.*, 1930, **46**, 290.

51. Yoshimatsu, S. I.: A new colorimetric urea method with 0.1 cc. of blood. *Tôhoku J. Exptl. Med.*, 1929, **13**, 1.
Chem. Abstracts, 1929, **23**, 4490.

CHAPTER XII

Urinary Ammonia

DISCUSSION OF PRINCIPLES

The concentration of ammonia in the blood is so slight and so constant that its determination is of no importance for clinical purposes. The reader is, therefore, referred for blood ammonia methods to the original sources, which have been reviewed by Nash and Benedict (8) and are discussed in the chapter on ammonia of volume I.

Determination of ammonia in urine has, in all methods that have become general, consisted of two steps, 1, removal of the ammonia from the urine and, 2, determination of the isolated ammonia.

Removal of ammonia from urine

Methods for accurate determination of ammonia in urine represent historically a succession of procedures for separating the ammonia from the urine without forming additional amounts by decomposition of other urinary substances. Boiling with even weak alkali can not be used because it decomposes urea into ammonia. Consequently methods were required which would affect the separation without heat. Four such procedures have been in vogue since 1850.

1. Schloesing's procedure for slow volatilization in a closed chamber (10). The urine was rendered alkaline by addition of lime, and was placed under a bell jar in a flat dish on top of which was a triangle. The triangle supported another dish which contained standard acid. Forty-eight hours were allowed for the ammonia to pass from the urine through the air to the acid. The method was much in use for fifty years. Shaffer, however, in 1902 (11) showed that the transfer of preformed ammonia was not complete, and that slow decomposition of urea occurred. Consequently it was possible to obtain results too low from incomplete transfer or too high from urea decomposition. When results were exact it was due to a balancing of errors.

2. Distillation in vacuo with sodium carbonate was introduced in 1850 by Boussingault (1), the ammonia being caught and titrated in standard acid. Various modifications of Boussingault's apparatus were used, but Shaffer (11) concluded that none was an improvement. He found that the

original method gave exact results, and that if alcohol were added to accelerate the distillation the latter could be completed in fifteen minutes.

3. Folin (2) in 1902 introduced a new principle, that of driving the ammonia by an air current from the urine into standard acid. The urine was made mildly alkaline by addition of about 4 per cent of sodium carbonate, and was nearly saturated with salt to decrease the solubility of the ammonia. The accuracy of this procedure was confirmed by Shaffer (11) who found that about 900 liters of air driven through 25 cc. of urine in ninety minutes removed all the ammonia. Van Slyke and Cullen (14) modified the procedure by using the highly soluble potassium carbonate both to set the ammonia free and to decrease its solubility. They found that if enough carbonate were added to make a solution half saturated or stronger, all the ammonia could be driven out of urine by 75 liters of air and in as short a time as five minutes, although it was usually more convenient to use a less violent stream and ten or fifteen minutes for the passage of the 75 liters.

4. Folin also introduced the next innovation. In 1917 with Bell (5) he showed that a complex aluminum-sodium silicate, which has become known as *permutit*, had the power to absorb quantitatively ammonia from neutral or slightly acid solutions, and to give it off again to alkali solution. With this solid reagent it was possible to separate the ammonia from urine by merely shaking for a few minutes. The ammonia could be regained in solution by equally short contact of alkali solution with the permutit-ammonia combination.

Present methods for isolation of ammonia from urine are based either on Folin's aeration principle or on his application of permutit, which have displaced the inaccurate and prolonged Schloesing method and the accurate and rapid but more complicated vacuum distillation.

Procedures for determination of ammonia after isolation from urine

For this determination three procedures have found application.

1. *Titration.* The ammonia removed from urine by aeration or distillation is caught in standard acid, and the excess acid is measured by titrating with standard alkali. This procedure has been described in connection with the Kjeldahl method in a preceding chapter.

2. *Colorimetric estimation with Nessler's solution.* This procedure has been described on page 533 for micro-Kjeldahl analyses.

3. *Gasometric determination with hypobromite.* This procedure has been described on page 354 of the chapter on gasometric methods. It can be applied either to the acid solution obtained when ammonia is aerated or distilled into excess acid, or to the alkaline solution obtained by the permutit method.

Of the three methods the titration has enjoyed the longest and widest use. Its exactness is limited only by the accuracy of standard solutions, volumetric measurements, and manipulation. Even in micro analyses the error can be kept without great difficulty below 1 per cent.

The gasometric determination is also of ancient lineage, although for some time neglected until recently revived for use with the manometric apparatus. It also is capable of accuracy better than 1 per cent, and it is independent of standard solutions.

Nesslerization as elaborated by Folin is a practical, rapid, and convenient method. The colorimetric error is 2 or 3 per cent.

The choice of one of these methods depends upon the method used to isolate the ammonia from the urine and on the degree of accuracy required by the object of the determination. The amount of ammonia present is sufficient for any of the three analyses.

If the ammonia is isolated by aeration it can be conveniently caught in standard acid and titrated directly in the receiving tubes.

When the permutit method is used, however, the isolated ammonia is extracted from the permutit by means of 10 per cent NaOH solution. The resultant alkaline solution can not be titrated, but is ready to be nesslerized without even pouring it off the permutit powder. It is seldom that an error of 2 or 3 per cent is of importance in the clinical interpretation of a urinary ammonia. The nesslerization procedure has therefore been usually employed when permutit has been used.

Procedures for determination of ammonia directly in urine without isolation

An adaptation of the Soerensen formol titration of amines was applied by Malfatti (7) in 1908 to determination of ammonia in urine. The method was based on the fact that when formaldehyde acts on a neutral ammonium salt neutral hexamethylene is formed and the acid previously combined with the ammonia is set free.

$$4\ NH_4Cl + 6\ CH_2O = N_4\ (CH_2)_6 + 6\ H_2O + 4\ HCl.$$

The acid thus freed can be titrated. However, other amines, notably amino acids, are also present in urine and react in a similar manner. Consequently the titration represents not ammonia, but ammonia + amines. Steel (12) has found that in ordinary urines the results are 10 to 20 per cent too high for ammonia. The Malfatti titration enjoyed, because of its simplicity and convenience, a considerable vogue in clinical laboratories, but appears now to be passing into deserved oblivion.

Sumner (13) uses nesslerization after interfering substances have been removed by precipitating cupric hydroxide in the urine.

Orr (9), without any preliminary treatment of the urine, adds phenol and sodium hypochlorite, which react with ammonia to form an intensely blue substance suitable for micro-determination of ammonia. Orr states that he was able to fix conditions such that neither the urea nor amino acids in the proportions present interfered with the accuracy of the results.

Neither of these direct colorimetric methods appears yet to have gained general use. Whether they equal or surpass Folin's permutit-nesslerization procedure in convenience or accuracy the writers can not state.

Precautions in handling urine

In order that ammonia determinations may be of any significance certain precautions in handling the urine must be observed. Otherwise ammonia may be lost from or formed in the urine in such quantities that the errors from these sources exceed the grossest inaccuracies likely to occur in analysis. If urine is alkaline ammonia can escape from it by volatilization. On the other hand bacterial growth may cause rapid ammonia formation from urea and other nitrogenous compounds. It is therefore essential to keep the urine acid and to prevent bacterial decomposition. If only ammonia estimations are required both objects are attained by adding concentrated sulfuric or hydrochloric acid as soon as the urine is collected, and storing the specimens in a refrigerator until they can be analyzed. Prolonged storage is to be avoided even in this case, since urea undergoes slow hydrolysis in acid solution even when cold.

If, as is often the case, titrations of urinary acidity are desired as well as ammonia values, the acid treatment can not be used. In this case the only way to be certain of reliable results is to analyze the urine within three or four hours at longest after it is passed. This is especially necessary if one intends to determine the ratio of ammonia to titratable acid, since transformation of a small part of the urea into ammonia would increase this ratio greatly.

If immediate analysis is impossible, one must make every attempt to avoid excessive contamination of the urine by using specially cleaned urinals. The urine is covered with a small amount of toluol as soon as it has been collected; the container is stoppered and placed immediately in the refrigerator. Analyses are made as early as possible, never more than twenty-four hours after the urine has been voided.

AERATION AND TITRATION METHOD OF FOLIN (2, 3, 4) AS MODIFIED BY VAN SLYKE AND CULLEN (14)

The method has already been described on page 549 of the urea chapter. Ordinarily the sample taken is 5 cc. of urine, as there directed, but of urine passed in severe diabetic acidosis only 2 cc. are taken, plus 3 cc. of water.

Calculation

$$0.34\ (A - B - C) = \text{milligrams of ammonia in sample.}$$

$$0.28\ (A - B - C) = \text{milligrams of ammonia nitrogen in sample.}$$

$$\frac{0.34\ (A - B - C)}{V} = \text{grams of ammonia per liter urine.}$$

$$\frac{0.28\ (A - B - C)}{V} = \text{grams of ammonia nitrogen per liter urine.}$$

$$\frac{20\ (A - B - C)}{V} = \text{milli-equivalents ammonia per liter urine.}$$

A = cubic centimeters 0.02 N acid placed in receiver; B = cubic centimeter 0.02 N alkali used in the titration; C = value of $A - B$ found in blank analysis of reagents, V = cc. urine in sample.

ABSORPTION WITH PERMUTIT AND NESSLERIZATION. FOLIN AND BELL (5)

Reagents

A 10 per cent solution of sodium hydroxide.

Nessler's solution and standard ammonia solutions prepared as described on pages 532 and 533 in the chapter on nitrogen.

Permutit. This is an insoluble sodium aluminum silicate (zeolite) discovered by Gans (6). It contains sodium which is easily replaced by ammonia. It is by means of this chemical replacement that the substance is able to absorb ammonia from solutions. The absorption occurs best in neutral or weakly acid solutions. In strongly alkaline solutions not only is no ammonia absorbed, but any which has been absorbed is given off again to the solution.

The reaction by which permutit absorbs ammonia is a reversible one, which becomes approximately complete only under definite and restricted conditions. Not only does alkali prevent the absorption: neutral salts also interfere with it, although to a less extent. To obtain approximately complete absorption of ammonia, salts must be present only in slight concentration, and a great excess of the permutit must be used. The presence of 0.1

gram of NaCl under the conditions given below for absorption of urinary ammonia is sufficient to prevent the process from being quantitative.

The removal of absorbed ammonia from permutit by means of alkali solution requires a definite time. Folin and Bell stated that with their preparation of permutit 95 per cent of absorbed ammonia went into solution in two to three minutes when extracted with 10 per cent NaOH, and that the extraction was complete in ten to fifteen minutes. They state, however, that different preparations vary in the speed with which they give up ammonia, and that one must determine by test how much time this requires. The tenacity with which the ammonia is held also increases with the length of time it has remained on the permutit. If absorbed ammonia has been left on the powder over night extraction is slower, and 2 or 3 per cent of the ammonia may be incapable of extraction.

The permutit can be used repeatedly. It can be freed of ammonia by washing with 10 per cent NaOH, then washed with 2 per cent acetic acid, by decantation, and finally with water, and air dried without heat. The solid powder contains about 20 per cent of combined water, and if this is driven off the activity of the powder is greatly reduced. It is an economical practice to collect used portions of the powder in a jar, and eventually purify them for use again.

The essential mechanical feature of permutit for ammonia determinations is that it is a clean, moderately fine insoluble powder, which gives off no dust or turbid material when shaken with water, but settles like sand in the course of a few seconds leaving a clear supernatant solution. Folin and Bell recommend a powder which will pass through a 60-mesh sieve but not through an 80-mesh one.

In case the powder as obtained yields a turbid suspension when shaken with water it is freed from dust by repeatedly shaking with water and decanting the turbid matter which does not settle quickly.

The activity of the powder *must be tested* by performing determinations with it on known solutions of ammonium sulfate or chloride of ammonia content similar to that found in urine, e.g., with 1 mg. of ammonia nitrogen per cubic centimeter.[1]

Procedure with permutit

Place about 2 grams of permutit in a 200-cc. volumetric flask and add about 5 cc. of water (no more). With an Ostwald pipette introduce 1 or 2 cc. of urine. With urines extraordinarily poor in ammonia it

[1] Permutit made according to Professor Folin's directions can be obtained from The Permutit Company, 440 Fourth Avenue, New York.

may be necessary to use 5 cc. Folin and Bell state, however, that it is preferable not to use more than 2 cc., since larger portions may introduce salts in sufficient quantity to prevent complete absorption of the ammonia. The added urine is rinsed down to the bottom of the flask with 1 to 5 cc. of water. The flask, containing the powder and 10 or 12 cc. of fluid, is shaken gently for five minutes to cause absorption of the ammonia. Add 25 to 40 cc. of water, mix gently with the powder, and decant after the latter has settled. Wash by decantation in the same way once more. If the urine contains bile two additional washings may be needed. After the last washing add a little water to the powder, then 5 cc. of the 10 per cent sodium hydroxide. Mix, then add more water till the flask is about three-fourths full. Shake a few seconds and then add 10 cc. of Nessler's reagent. Mix, let stand for at least ten minutes. Finally fill up to the mark with water, mix again, and compare the supernatant solution in a colorimeter with a standard containing 0.5 or 1.0 mg. of ammonia nitrogen.

The standard is made by placing in a 200 cc. flask 5 or 10 cc. of stock solution containing 1 mg. of ammonia nitrogen per 10 cc. Five cc. of 10 per cent sodium hydroxide are added, then water and Nessler's solution as in the flask with the urine.

Calculation

$$\frac{n\ S}{V\ U} = \text{grams of ammonia nitrogen per liter urine.}$$

$$\frac{1.214\ n\ S}{V\ U} = \text{grams of ammonia as } NH_3 \text{ per liter urine.}$$

$$\frac{71.4\ n\ S}{V\ U} = \text{milli-equivalents } NH_3 \text{ per liter urine.}$$

S and U represent the readings in the colorimeter of the standard and unknown respectively, V represents the volume in cubic centimeters of the urine sample taken for the analysis, and n is the number of milligrams of ammonia nitrogen in the standard solution.

ALTERNATIVE DISTILLATION AND TITRATION PROCEDURE AFTER PERMUTIT ABSORPTION OF AMMONIA

The urine is treated through the point where the 10 per cent sodium hydroxide is introduced, exactly as above described, except that the procedure is carried out in a small Kjeldahl flask. After alkali and a

little water have been added to the mixture the ammonia is distilled from the alkali-permutit mixture, as described for micro-Kjeldahl determinations in chapter X, preferably with steam distillation.

BIBLIOGRAPHY

1. Boussingault, von: Ueber die im Harne enthaltenen Mengen von Ammoniak. *Ann. de. chim. et de phys.*, 1850, **29**, 472.
2. Folin, O.: Eine neue Methode zur Bestimmung des Ammoniaks im Harne und andere thierischen Flüssigkeiten. *Zeit. physiol. Chem.*, 1902–3, **37**, 161.
3. Folin, O.: Approximately complete analysis of thirty "normal" urines. *Am. J. Physiol.*, 1905, **13**, 45.
4. Folin, O.: Note on the determination of ammonia in urine. *J. Biol. Chem.*, 1910–11, **8**, 497.
5. Folin, O., and Bell, R. D.: Applications of a new reagent for the separation of ammonia. I. The colorimetric determination of ammonia in urine. *J. Biol. Chem.*, 1917, **29**, 329.
6. Gans, R.: Zeolithe und ähnliche Verbindungen, ihre Konstitution und Bedeutung für Technik und Landwirtschaft. *Jahrbk. preuss. Geol. Landesanstalt.*, 1905, **26**, 179.
   Konstitution der Zeolithe, ihre Herstellung und technische Verwendung. *Jahrbk. preuss. Geol. Landesanstalt.*, 1906, **27**, 63. (Cited by Folin and Bell (5).)
7. Malfatti, H.: Eine Klinische Methode zur Bestimmung des Ammoniaks im Harne. *Z. anal. Chem.*, 1908, **47**, 273.
8. Nash, T. P., Jr., and Benedict, S. R.: The ammonia content of the blood and its bearing on the mechanism of acid neutralization in the animal organism. *J. Biol. Chem.*, 1921, **48**, 463.
9. Orr, A. P.: A colorimetric method for the direct estimation of ammonia in urine. *Biochem. J.*, 1924, **18**, 806.
10. Schlösing, Th.: Über die Bestimmung des Ammoniaks und namentlich über die Bestimmung desselben in Tabak. *Chem. Centralblatt*, 1851, **22**, 432.
    Ann. de chim et de phys., 1851, Series 3, **31**, 153.
    Journ. f. prakt. Chem., 1851, **52**, 372.
11. Shaffer, P. A.: On the quantitative determination of ammonia in urine. *Am. J. Physiol.*, 1902–3, **8**, 330.
12. Steel, M.: An improvement of the Folin method for the determination of urinary ammonia nitrogen. *J. Biol. Chem.*, 1910, **8**, 365.
13. Sumner, J. B.: A new method for the direct nesslerization of ammonia in urine. *J. Biol. Chem.*, 1918, **34**, 37.
14. Van Slyke, D. D., and Cullen, G. E.: The determination of urea by the urease method. *J. Biol. Chem.*, 1916, **24**, 117.
    A permanent preparation of urease, and its use in the determination of urea. *J. Biol. Chem.*, 1914, **19**, 211.

CHAPTER XIII

Amino Acids

GENERAL METHODS FOR AMINO NITROGEN DETERMINATION

The formol titration of Sörensen

The first method proposed for the determination of amino acids in blood of urine was that of Sörensen (7). If to a neutral salt, $RNH_2 \cdot HA$, of a primary amine with an acid, HA, neutral formaldehyde solution is added, the reaction,

$$RNH_2 \cdot HA + OCH_2 = RN : CH_2 + H_2O + HA,$$

takes place and the acid previously neutralized by the amino group is set free. The amount of alkali required to titrate the solution back to the neutral point is a measure of the amount of amine present. The reaction occurs with ammonium salts as well as with primary amines, so that before the amines can be determined in biological fluids ammonium salts must be removed. The formaldehyde titration was applied by Henriques and Sörensen (4) to measurement of the amino acid nitrogen of urine. The coloring matter of the urine complicated the procedure considerably. Perhaps for this reason the ingenious and elegant method of Sörensen has not gained great use in urine analysis. Its accuracy is also diminished by the presence of buffers, since it is necessary to bring the solution to pH 7 before adding the formaldehyde, but to pH 9 for the end point of the final titration. Any buffers present which are active over this pH range are titrated as amino acids. The formaldehyde titration apparently has never been applied to clinical blood analyses, presumably because it is not sensitive enough to determine the slight amounts of amino nitrogen in the usual blood filtrates. Because of the difficulties involved in its application to blood and urine, the formol method will not be described.

The gasometric nitrous acid method of Van Slyke

Another reaction that has been applied to determination of primary amino nitrogen is that with nitrous acid, the N_2 gas evolved being measured.

$$RNH_2 + HNO_2 = ROH + H_2O + N_2$$

This was employed in 1912 by Levene and Van Slyke (6) for analysis of urine and by various authors, using the Van Slyke amino nitrogen apparatus, to blood analysis (1, 5). Because the method required a special apparatus and a somewhat complicated previous preparation of the blood, its use was limited to chemical and physiological studies, and it did not become a clinical procedure. Later, however, Van Slyke (8) adapted the nitrous acid reaction to use with the manometric gas apparatus, which permits the determination to be made directly on the Folin-Wu filtrate.

The colorimetric method of Folin

The third method to find general application in biochemistry has been the color reaction of Folin (2) with β-napthoquinonesulfonate.

The methods here described are the Folin colorimetric and the Van Slyke gasometric. Both can be carried out on 5 cc. of Folin-Wu blood filtrate. The gasometric is completed in fifteen minutes, while the colorimetric requires a number of hours. This difference in time is ordinarily not important because a series of samples can be prepared together for the colorimeter, and during most of the time consumed they require no attention from the analyst.

FOLIN'S COLORIMETRIC METHOD FOR AMINO ACIDS IN URINE (3)

Reagents

Standard amino acid solutions can be made from glycine, glutamic acid, leucine, phenyl alanine or tyrosine. Of these Folin prefers glycine. The glycine is dissolved in 0.1 N hydrochloric acid containing 0.2 per cent sodium benzoate as a preservative.

A *stock standard solution of glycine*, which serves also as the *regular standard for urine analysis*, is made as follows. Dissolve 536 mg. of glycine and 2 grams of sodium benzoate in 0.1 N hydrochloric acid in a liter flask. Dilute up to 1 liter with 0.1 N hydrochloric acid. The solution contains 0.1 mg. of amino acid nitrogen per cubic centimeter.

Dilute standard for blood analysis. Dilute 70 cc. of the stock standard to 100 cc. with 0.1 N hydrochloric acid, to obtain a solution with 0.07 mg. of amino acid nitrogen per cubic centimeter.

One per cent sodium carbonate solution. Fifty cubic centimeters of saturated sodium carbonate solution are diluted to 500 and the concentration is so adjusted that 8.5 cc. of this solution are equivalent to 20 cc. of 0.1 N hydrochloric acid when titrated with methyl red as indicator.

Fresh 0.5 per cent solution of sodium β-naphthoquinonesulfonate: One hundred milligrams weighed within 5 per cent are dissolved in 20 cc. of

water. The solution darkens in a few hours, and must, therefore, be used at once. The β-naphthoquinonesulfonate is on the market (Eastman Kodak Company) but may be prepared in the laboratory by directions given in detail by Folin (3).

Acetic acid, acetate solution. Mix 100 cc. of 50 per cent acetic acid with an equal volume of 5 per cent sodium acetate solution.

A 4 per cent solution of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$). This is used to destroy the surplus quinone remaining after the amino acid color has developed.

Permutit, prepared and tested as on pages 577–578.

Phenolphthalein, 0.25 per cent in 50 per cent alcohol.

Procedure

It is necessary first to remove ammonia by means of permutit.

Dilute 5 to 25 cc. of urine (enough to contain 0.3 to 2.0 mg. of amino acid nitrogen) to 25 cc. in a 50 cc. Erlenmeyer flask. Add 2 to 3 grams of permutit and agitate gently, but continuously for five minutes. Decant the supernatant urine into another 50 cc. flask; again add 2 to 3 grams of permutit and shake as before for five minutes. Decant the supernatant urine into a flask or test tube. A little turbidity at this point does not interfere with the determination.

To test tubes graduated at 25 cc. add 1, 2 and 3 cc. respectively of the standard glycine solution for urine and equal amounts of the special 1 per cent sodium carbonte (i. e., 1 cc. of sodium carbonate for each cubic centimeter of 0.1 N hydrochloric acid). To another similar tube transfer 5 cc. of the ammonia-free urine followed by 1 cc. of 0.1 N hydrochloric acid and 1 cc. of sodium carbonate. Dilute the contents of each test tube to 10 cc. Dissolve 250 mg. of sodium β-naphthoquinonesulfonate in 50 cc. of water, and add 5 cc. of this solution to each standard and to the unknown urine.

Mix and set in a dark place. After the tubes have stood ten to fifteeen minutes it is advisable to take them out for inspection. If the tube containing the urine appears much darker than the darkest standard, it is necessary to start again with another smaller sample of urine. The unknown must always be made up to a final volume of 15 cc. like the standard. The tubes are returned to the dark and allowed to stand over night.

The following day standards and unknowns are acidified to phenolphthalein by adding acetic acid-acetate solution. To each tube are then added 5 cc. of 4 per cent sodium thiosulfate and water to make

25 cc. After mixing, the color of the unknown is compared, in a colorimeter, with the standard most nearly resembling it.

Calculation

$$\frac{0.1\ SQ}{VU} = \text{grams of amino acid nitrogen in a liter of urine.}$$

S and U = readings of standard and unknown respectively, V = the cubic centimeters of undiluted urine taken for the final analysis, and Q = the number of cubic centimeters of stock standard glycine solution used in the standard.

FOLIN'S (2) COLORIMETRIC METHOD FOR AMINO ACIDS IN BLOOD

Procedure

The determination is made preferably with 10 cc. of Folin-Wu tungstic acid filtrate, but may be made with 5 cc. (See precipitation of proteins, p. 65.)

With 5 cc. of filtrate. Transfer to a test tube with a graduation mark at 30 cc., 1 cc. of the standard glycine solution and add 3 cc. of water. To a similar tube with a mark at 15 cc. add 5 cc. of blood filtrate. Add 1 drop of 0.25 per cent phenolphthalein solution to each. Add 1 cc. of the 1 per cent sodium carbonate solution to the standard and then add to the filtrate, drop by drop, enough carbonate solution to give it the same pink color as the standard.

Add 5 cc. more of water to the standard, which is to have twice the volume of the blood filtrate. Then prepare a fresh 0.5 per cent solution of the sodium β-naphthoquinonesulfonate. Add 2 cc. of this to the standard and 1 cc. to the blood filtrate. Shake the tubes a little to mix the solutions, and set them in a completely dark place till the following day, nineteen to thirty hours.

At the end of this time add 2 cc. of the acid acetate solution to the standard and 1 cc. to the blood filtrate. ***After*** the introduction of the acid acetate-solution, add 2 cc. of the thiosulfate solution to the standard and 1 cc. to the blood filtrate. Finally dilute the standard to 30 cc., and the blood filtrate to 15 cc. with water. Mix and make the color comparison, setting the standard at 20 mm.

If ***10 cc. of the Folin-Wu blood filtrate*** are available the procedure is midified in the following manner:

Two test tubes marked to contain 25 cc. are used. In one place 1 cc. of standard glycine solution plus 8 cc. of water. In the other place

10 cc. of filtrate. Add a drop of phenolphthalein solution. Add 1 cc. of the sodium carbonate solution to the standard, and enough to the filtrate to give it the same pink color. Add 2 cc. of freshly prepared β-naphthoquinonesulfonate solution to each, mix, and let stand in the dark till next day. Then add to each 2 cc. of the acetic acid-acetate solution followed by 2 cc. of the thiosulfate. Dilute each to the 25 cc. mark and compare the colors.

Results

$$\frac{7\,S}{U} = \text{milligrams of amino acid nitrogen per 100 cc. of blood.}$$

S = depth of standard solution. U = depth of unknown.

GASOMETRIC METHODS FOR BLOOD AND URINE

These methods are described on page 385 in the chapter on gasometric methods.

BIBLIOGRAPHY

1. Bock, J. C.: The estimation of amino-acid nitrogen in blood. *J. Biol. Chem.* 1916–17, **28**, 357.
2. Folin, O.: A system of blood analysis. Supplement III. A new colorimetric method for the determination of the amino-acid nitrogen in blood. *J. Biol. Chem.*, 1922, **51**, 377.
3. Folin, O.: A colorimetric determination of the amino acid nitrogen in normal urine. *J. Biol. Chem.*, 1922, **51**, 393.
4. Henriques, V., and Sörensen, S. P. L.: Über die quantitative Bestimmung der Aminosauren, Polypeptide, und die Hippursäure im Harne durch Formoltitration. *Z. physiol. Chem.*, 1909, **63**, 27.
5. Hiller, A., and Van Slyke, D. D.: A study of certain protein precipitants. *J. Biol. Chem.*, 1922, **53**, 253.
6. Levene, P. A., and Van Slyke, D. D.: Gasometric determination of free and conjugated amino acids in the urine. *J. Biol. Chem.*, 1912, **12**, 301.
7. Sörensen, S. P. L.: Enzymstudien. *Biochem. Z.*, 1908, **7**, 45.
8. Van Slyke, D. D.: Manometric determination of primary amino nitrogen and its application to blood analysis. *J. Biol. Chem.*, 1929, **83**, 425.

CHAPTER XIV

Uric Acid

DISCUSSION

Until the era of Folin's colorimetric micro methods clinical determinations of uric acid were limited to the urine; the amounts present in blood were too small to be analyzed by the means available. From 1875 to 1892 the accepted procedure for urine was that of Salkowski and Ludwig (30), in which the uric acid was precipitated with ammoniacal silver nitrate and magnesium chloride, the precipitate was decomposed with hydrogen sulfide, and the free uric acid was crystallized from hydrochloric acid and weighed. In 1892 Hopkins (22) showed that uric acid could be quantitatively precipitated by ammonium salts, particularly by saturation with ammonium chloride, and could be titrated in the redissolved precipitate by means of permanganate. Hopkins' method as modified by various authors (8, 16, 31) remains the accepted volumetric analysis for uric acid in urine.

The formation of blue color when tungstic acid and uric acid come into contact in solution forms the basis of all uric acid methods that have attained general use in blood analysis. Frabot (19) in 1905 noted that uric acid added to alkaline tungstate solution developed a color so intense that uric acid in 1 part per 10,000 of solution could be detected. It was not, however, until Folin with Macallum (14) and Denis (13) in 1912 studied the conditions of the reaction that its application began to blood analysis. The formation of the color is due to the same reduction of tungstic acid to blue reduction products which is utilized in the Folin-Benedict copper blood sugar methods. In the sugar methods the reduction is effected by cuprous salts under conditions which minimize the effect of uric acid. In the uric acid method no such reducing salts are present, and conditions are arranged to obtain the maximum reducing effect of uric acid.

The original Folin-Denis (13) method required 20 to 25 cc. of blood, and was time consuming. The proteins were precipitated by heat and acetic acid, the filtrate was boiled down, and the uric acid was precipitated by silver lactate, magnesia, and ammonia. The precipitate was redissolved and the phosphotungstic acid added. The method was at once widely applied, and numerous difficulties were encountered with it. The chief sources of error were found to lie in: 1, the procedures for precipitation of proteins; 2, the quantitative separation of uric acid from other blood constituents which either inhibit the uric acid reaction or (as the phenols) themselves react so that errors may be either

positive or negative; 3, difference between blood filtrate and standard solution in rate of color development; 4, turbidity formation in the course of color development; 5, the faintness of the color produced; 6, the difficulty of preparing stable standards.

Most of these difficulties have been overcome by the work of Folin, Benedict, and others. Avoidance of heat and high acidities (21, 28) was found necessary to prevent loss of uric acid in protein coagulation, and these aims were attained by the Folin-Wu tungstic acid precipitation (18).

Hunter and Eagles (23) and Benedict, Newton, Behre and Davis (6) have succeeded in isolating from the cells of blood what appears to be the most important interfering substance. This substance, thioneine, a sulfur-containing compound, is not found in serum or plasma, and its concentration in the blood cells appears to diminish in nephritis and other conditions in which uric acid accumulates in the blood. Its variations in health and disease have, as yet, been little investigated. It gives a color with all the tungstate uric acid reagents.

For *plasma* analyses the inaccurate and time consuming procedure of isolating the uric acid by precipitation with the ammonium-magnesium-silver reagent was obviated by Benedict (2), who devised in his arseno-phosphotungstate used with cyanide a color reagent so specific for uric acid that other constituents of plasma filtrates do not interfere.

In *whole blood*, however, the presence of thioneine, and perhaps other interfering substances, still renders preliminary purification necessary before the color reagent can be used. In place of the original ammonium-magnesium-silver precipitating reagent for uric acid, silver lactate (12, 18), zinc salts (25, 26, 27), and nickel salts (9) have been used with more satisfactory results. However, Folin (12) in the most recent application of such methods, using silver lactate, recovered only 60 to 80 per cent of added uric acid, and nevertheless obtained results that he considered too high when he analyzed blood low in uric acid content. When uric acid was high incomplete recovery made the results low, and when uric acid was low other precipitable color-forming substances from the cells made the results too high, correct analyses being obtained only when the errors balanced. Benedict (4) has introduced a new idea into the technique for isolating uric acid from cellular interfering substances. He finds that the chief of the latter, thioneine, is completely removed when silver chloride is precipitated in *strongly acidified* blood filtrate, while uric acid remains in solution. He accordingly simplifies the preliminary treatment of the blood filtrate to the process of precipitating silver chloride in it and centrifugating, the supernatant filtrate without further treatment being used for the colorimetric determination. Benedict (4) obtained with this procedure in analysis of whole blood results agreeing with those by the Folin-Wu (18) technique, in which the uric acid was precipitated with the silver chloride in slightly acid solution, and recovered by extraction of the precipitate with hydrochloric acid. It appears doubtful, from Folin's results (11), that any method now available for uric acid determination in whole blood is accurate. The recent Benedict (4) procedure appears, however, to be as accurate as the more complicated previous methods, and excels them in simplicity and rapidity.

As a departure from colorimetric blood uric acid analyses Curtman and Lehrman (9) devised a method in which the uric acid was precipitated by nickle acetate and titrated iodometrically. Recovery of uric acid added to even whole laked blood was quantitative. The method requires 10 cc. of blood, and is more time consuming than the direct colorimetric procedures

Presumably for these reasons it has never received sufficiently extensive use to indicate how satisfactory it is for general work.

The interfering substances that have caused so many difficulties with colorimetric uric acid determinations in blood do not apparently occur in disturbing proportions in urine. Either the Benedict or Folin colorimetric reagents can be applied directly to urine.

URIC ACID IN URINE. COLE'S (8) MODIFICATION OF HOPKINS' TITRATION METHOD (22)

The urine is treated with colloidal iron to remove an unknown substance that is precipitable by ammonium chloride. The filtrate is treated with solid ammonium chloride and made strongly alkaline with ammonia. The uric acid is rapidly and quantitatively precipitated as ammonium urate. This is filtered off, washed with ammonium sulphate to remove the greater part of the chlorides, dissolved in hot sulfuric acid, and titrated with standard potassium permanganate. The end point is reached when a momentary pink flush is seen over the whole body of the fluid. This marks a stage when the rate of oxidation of the uric acid suddenly decreases. Up to this point 1 cc. of 0.05 N permanganate is found empirically to correspond to 3.7 mg. of uric acid. The chemical changes involved in the oxidation have not yet been determined.

Reagents

Colloidal (dialysed) iron, 0.6 per cent.

Pure, dry, recrystallised ammonium chloride.

Washing fluid. One hundred grams of pure ammonium sulfate are dissolved in about 800 cc. of distilled water, 10 cc. of strong ammonium are added and the volume made up to 1 liter with water. It is convenient to use this from a wash bottle with a fine jet.

Forty-five per cent sulfuric acid (by volume). To 500 cc. of distilled water in a large flask cautiously add 450 cc. of pure concentrated sulfuric acid, cooling at intervals. Cool thoroughly under the tap and make up the volume to 1000 cc.

0.05 N potassium permanganate. (See p. 31.)

Procedure

If the urine contains much albumin it is removed by coagulation with heat and acetic acid. Small amounts are removed during the routine colloidal iron treatment.

To 150 cc. of urine, or the filtrate from coagulated albumin, add 30 cc. of the colloidal iron, stirring well during the addition. Filter through two dry papers into two dry flasks (two being used to save time). When at least 100 cc. of the filtrate has been collected, measure in a cylinder the amount taken and transfer it to a beaker, which has been previously washed and drained. It is convenient to take 100 cc., but with dilute urines it is better to take 120 or 150 cc. For every 10 cc. of the filtrate taken weigh out 2 grams of the solid ammonium chloride; add this to the beaker and stir well. When it has dissolved add 3 cc. of concentrated ammonia and stir again. Stir at intervals for 20 minutes, then remove the rod and let it rest on the rim of the beaker.

When the bulk of the precipitate has settled the urate is filtered off, either through plain paper or through an asbestos mat of 20 or 25 mm. diameter in a perforated porcelain funnel or in a Gooch crucible. Use only moderate suction. When the main mass of the filtrate has passed through, transfer the bulk of the precipitate to the mat, but do not suck dry. Wash out the precipitate remaining in the beaker with *the ammonium sulfate solution on to the filter and start suction again.* Do this twice more, finally sucking the precipitate dry. The object of the washing is to remove the chloride from the precipitate, since chloride reacts somewhat with permanganate. If the filtration has been done on a paper, transfer it and precipitate to the beaker in which the urate was precipitated. If the washing was done on an asbestos mat, blow the mat from the inverted funnel or crucible into the beaker and wash with hot water any adherent particles of precipitate also into the beaker. Add hot water to make a total volume of 100 cc., preparatory to titration. Add 20 cc. of the 45 per cent sulfuric acid and stir with a thermometer. The whole of the precipitate must be dissolved, if necessary by the aid of heat, before the titration is commenced. Cool or heat to 65°C. Titrate with the standard permanganate. The permanganate must be added rather slowly, with constant stirring. The end point is reached when the addition of a couple of drops causes a *faint* pink flush through the whole body of the fluid. A very considerable addition has to be made for the pink to be permanent, but the empirical valuation of the permanagnate is based on the first temporary flush throughout the solution as the end point.

Calculation

Each cubic centimeter of permanganate solution corresponds to 3.7 mg. of uric acid. Therefore, when the material titrated represents V cubic

centimeters of urine and requires A cubic centimeter of 0.05 N permanganate, the calculation is made by the following formulae:

$$\frac{3.7\ A}{V} = \text{grams of uric acid in 1 liter urine.}$$

$$\frac{1.23\ A}{V} \quad \text{grams of uric acid nitrogen in 1 liter urine.}$$

V = five-sixths the cubic centimeter volume of urine filtrate used.

URIC ACID IN URINE. COLORIMETRIC METHOD OF BENEDICT AND FRANKE (5)

Reagents

Sodium cyanide solution, 5 per cent, containing 2 cc. of concentrated ammonia solution per liter. This solution should not be used after six weeks.

Arseno-phosphotungstic acid. Dissolve 100 grams of sodium tungstate in about 600 cc. of water in a liter flask. Add 50 grams of pure arsenic pentoxide followed by 25 cc. of syrupy phosphoric acid (85 per cent H_3PO_4) and 20 cc. of concentrated hydrochloric acid. Boil twenty minutes, cool and dilute to 1 liter. This reagent keeps indefinitely.

Benedict-Hitchcock stock standard solution, 0.2 mg. per cubic centimeter (6)

Dissolve 9 grams of pure, crystallized disodium-hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) and 1 gram of sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) in 200 to 300 cc. of hot water and filter if not perfectly clear. Make the solution up to 500 cc. with hot water, and pour this warm solution on exactly 200 mg. of uric acid, suspended in a few cubic centimeters of water in a liter volumetric flask. Shake gently until the uric acid is completely dissolved. Cool. Add exactly 1.4 cc. of glacial acetic acid, dilute to 1 liter and mix. Add 5 cc. of chloroform to prevent bacterial growth. It will keep in a cool place about two months.

Alternative standard uric acid solution of Folin, 1 mg. per cubic centimeter (12)

This standard has the advantage of keeping almost indefinitely. Place 1 gram of uric acid, weighed to 1 mg., in a liter volumetric flask. To 0.6 gram of lithium carbonate in a 250-cc. flask, add 150 cc. of water and shake until the salt is dissolved. Filter the solution to remove all traces of insoluble material. Heat the filtered solution to 60° and warm the flask containing the uric acid under warm running water. Pour the warm liquid into the flask, at the same time washing down crystals of uric acid adherent to the neck. Shake the warm mixture for five minutes, then cool the flask under running cold water without delay. (Slight turbidity may be neglected as it is not due to undissolved uric acid.) Add 20 cc. of formalin (40 per cent solution of formaldehyde) and half fill the flask with water. Add a few drops of methyl orange and finally introduce rather slowly and with shaking, by means of a pipette, 25 cc. of normal sulfuric acid. The solution

should turn pink while 2 or 3 cc. of acid are still in the pipette. Dilute the solution to a liter, mix it thoroughly and transfer it to a tightly stoppered bottle. It should be stored in the dark.

Urine standard uric acid solution. To 50 cc. of the Benedict-Hitchcock stock standard, or 10 cc. of the Folin standard, in a 500-cc. volumetric flask add 350 cc. of water and 25 cc. of 1:10 hydrochloric acid, made by 10-fold dilution of the concentrated acid. Dilute to the mark. Unless this diluted standard is kept in an atmosphere of carbon dioxide it must be made up fresh from the stock standard every two weeks. It contains 0.02 mg. of uric acid per cubic centimeter.

Procedure

Dilute the urine with water so that 10 cc. will contain 0.15 to 0.30 mg. of uric acid (a 1 to 20 dilution is usually satisfactory). To 10 cc. of the diluted urine, in a 50-cc. volumetric flask, add 5 cc. of the 5 per cent sodium cyanide solution, followed by 1 cc. of the arseno-phosphotungstic acid reagent. (***Both these solutions are highly poisonous and should be added from burettes, never from pipettes.***) Mix the solution by gentle shaking and, after it has stood five minutes, dilute it to the mark with water and mix it again. Ten cubic centimeters of the ***urine standard*** are simultaneously treated in the same manner in another flask. Standard and unknown are then compared in the colorimeter.

Calculation of results

$$\frac{0.2\ S}{U\ V} = \text{grams of uric acid in 1 liter of urine.}$$

$$\frac{0.067\ S}{U\ V} = \text{grams of uric acid nitrogen in 1 liter of urine.}$$

S = reading of standard, U = reading of unknown, V = the cubic centimeters of *undiluted* urine in the 10 cc. taken for analysis.

If the urine contains appreciable amounts of albumin this must be removed in advance by heat and acetic acid and filtration.

URIC ACID IN BLOOD PLASMA. BENEDICT'S DIRECT COLORIMETRIC METHOD (2, 3)

Reagents

Five per cent sodium cyanide.

Arseno-phosphotungstic acid, described above for colorimetric urine method.

Dilute standards for use in determinations of blood uric acid. 1. To 25 cc. (= 5 mg. uric acid) of the Benedict-Hitchcock stock uric acid solution, or 5 cc. of the Folin stock solution (both described above for the colorimetric urine method) in a 500 cc. volumetric flask, add about 200 cc. of water, 25 cc. of dilute hydrochloric acid (1 volume of concentrated HCl to 9 volumes of water) and dilute to 500 cc. with water. One cubic centimeter contains 0.01 mg. of uric acid.

2. A second standard, to contain 0.004 mg. of uric acid per cubic centimeter (0.02 mg. of uric acid in 5 cc.) is made by diluting 10 cc. of the Benedict-Hitchcock stock solution (or 2 cc. of the Folin stock solution) and 25 cc. of dilute hydrochloric acid to 500 cc. in the same way.

The diluted standards keep only one or two weeks if exposed to the air. Benedict suggests that they be kept in an atmosphere of carbon dioxide. This may be accomplished by placing the solution in a bottle fitted with a two-holed rubber stopper. Through one of these holes a glass tube passes almost to the bottom of the bottle. In the other hole is a short glass tube. To the outer ends of both tubes rubber connecting tubes are attached. The solution is introduced into the bottle, a Kipp CO_2 generator is attached to the longer tube and a current of CO_2 is passed through the solution until the air is completely driven from the liquid and the dead air space above it. The two rubber tubes are then closed with pinch-cocks and the CO_2 generator is transferred to the shorter tube, instead of the longer one. Solution can then be withdrawn at will from the longer tube through which it is delivered by the CO_2 pressure produced by the generator permanently attached to the bottle. Under these conditions Benedict states that even the dilute standard will keep a long time.

Procedure

To 5 cc. of Folin and Wu protein-free filtrate of plasma, or serum, or to the filtrate from *unlaked* whole blood (p. 67), in a large test tube, add 5 cc. of water. Treat 5 cc. of the dilute standard no. 2 (containing 0.02 mg. of uric acid per 5 cc.) in the same way in another tube. To each add 5 cc. of the sodium cyanide solution and then 1 cc. of the arseno-phosphotungstic acid reagent. (***Both of these solutions are highly poisonous, and should be added from burettes, never by means of pipettes.***) Mix at once by inverting the tubes, place the latter immediately in boiling water and allow them to stand in the boiling water for three minutes. At the end of this time remove them to a beaker of cold water and leave them three minutes more. Then remove them and read the unknown against the standard in a colorimeter.

If the standard is not of the proper strength to permit colorimetric comparison repeat the determination. If the unknown was too strong use the stronger standard, no. 1, containing 0.01 mg. of uric acid per cubic

centimeter. If the unknown was too weak use more blood filtrate for the second determination. The whole analysis must be repeated, because both standard and unknown must be treated at approximately the same time to obtain satisfactory results. For the same reason it is not advisable to attempt to analyze more than a limited number of blood samples at one time. It is not permissible to alter the final dilution of either standard or unknown.

Calculation

$$\frac{100\ S\ D}{V\ U} = \text{milligrams of uric acid in 100 cc. of blood.}$$

$$\frac{4\ S}{U} = \text{milligrams of uric acid in 100 cc. of blood, when } D = 0.02 \text{ and } V = 0.5.$$

S and U represent the readings of standard and unknown respectively, D = milligrams of uric acid in standard, V = cubic centimeters of blood represented in filtrate used.

URIC ACID IN EITHER PLASMA OR WHOLE BLOOD. COLORIMETRIC METHOD OF BENEDICT AND BEHRE (4) ON SILVER CHLORIDE FILTRATE

Thioneine, the chief interfering substance in whole blood, is removed by precipitation in acid solution with silver chloride The AgCl carries down with it all of the thioneine and none of the uric acid. The uric acid in the supernatant solution is determined as in the filtrate from blood plasma, analyzed as described above.

Reagents

Five per cent sodium cyanide, arseno-phosphotungstic acid color reagent, and a *uric acid standard solution containing 0.004 mg. uric acid per cubic centimeter*, as described above for the colorimetric urine analysis.

Lithium chloride solution. Three grams of lithium chloride and 20 cc. of concentrated hydrochloric acid made up to 1 liter with water.

Silver nitrate solution containing 11.6 grams of $AgNO_3$ per liter.

Procedure

The proteins from either whole blood or plasma are precipitated, either by the tungstic acid reagent of Folin and Wu (see p. 65) or the tungstomolybdic acid reagent of Benedict (see p. 68). In either case the blood is mixed with 9 volumes of solution, so that a 1:10 dilution is obtained. It is desirable to use sodium or lithium oxalate rather than

potassium oxalate as anticoagulant in the blood, because the potassium salt is more likely than either of the others to cause turbidity during the uric acid determination.

Of the 1:10 blood filtrate transfer 5 cc. to a 15-cc. centrifuge tube. Add 2.5 cc. of the lithium chloride solution and mix by inversion of the tube. Add 2.5 cc. of the silver nitrate solution, and shake the contents of the tube thoroughly, using a tight rubber stopper to close the tube. Centrifuge for thirty seconds or longer, and decant all the supernatant solution into a test tube. If the solution is slightly cloudy from the presence of a trace of colloidal silver chloride, it does not matter. The opalescence will disappear when cyanide is added.

In another test tube place 5 cc. of the standard uric acid solution and 5 cc. of water.

To each tube add 4 cc. of the sodium cyanide solution and 1 cc. of the color reagent. ***(Both solutions are intensely poisonous, and should be added from burettes, not from pipettes)*** Each tube is inverted once immediately after addition of the color reagent, and is placed at once in a boiling water bath, where the tubes are left three minutes after immersion of the last tube. The tubes are then removed from the bath, and are allowed to stand for about two minutes at room temperature. They are then read in a colorimeter while still warm or even hot. There is less likelihood of turbidity if the readings are made before the solutions have cooled. Even when excessive amounts of potassium oxalate have been used the turbidity is not likely to develope if the readings are made while the solutions are still warm.

Avoid breathing poisonous HCN fumes after adding NaCN to the acidified blood filtrate.

Calculation

$$\frac{4\,S}{R} = \text{milligrams of uric acid per 100 cc. blood.}$$

BIBLIOGRAPHY

1. Benedict, S. R.: On the colorimetric determination of uric acid in blood. *J. Biol. Chem.*, 1914–15, **20**, 629.
2. Benedict, S. R.: The determination of uric acid in blood. *J. Biol. Chem.*, 1922, **51**, 187.
3. Benedict, S. R.: The determination of uric acid. *J. Biol. Chem.*, 1922, **54**, 233.
4. Benedict, S. R., and Behre, J. A.: The analysis of whole blood. III. Determination and distribution of uric acid. *J. Biol. Chem.*, 1931, **92**, 161.

5. BENEDICT, S. R., AND FRANKE, E.: A method for the direct determination of uric acid in urine. *J. Biol. Chem.*, 1922, **52**, 387.

6. BENEDICT, S. R., AND HITCHCOCK, E. H.: On the colorimetric estimation of uric acid in urine. *J. Biol. Chem.*, 1914–15, **20**, 619.

7. BENEDICT, S. R.; NEWTON, E. B., AND BEHRE, J. A.: A new sulfur-containing compound (thiasine) in the blood. *J. Biol. Chem.*, 1926, **67**, 267.

8. COLE, S. W.: Practical physiological chemistry. 8th Ed., page 404, Cambridge, 1928.

9. CURTMAN, L. J., AND LEHRMAN, A.: A new volumetric method for the determination of uric acid in blood. *J. Biol. Chem.*, 1918, **36**, 157.

10. FOLIN, O.: A system of blood analysis. Supplement IV. A revision of the method for determining uric acid. *J. Biol. Chem.*, 1922, **54**, 153.

11. FOLIN, O.: Unlaked blood as a basis for blood analysis. *J. Biol. Chem.*, 1930, **86**, 173.

12. FOLIN, O.: An improved method for the determination of uric acid in blood. *J. Biol. Chem.*, 1930, **86**, 179.

13. FOLIN, O., AND DENIS, W.: A new (colorimetric) method for the determination of uric acid in blood. *J. Biol. Chem.*, 1912–13, **13**, 469.

14. FOLIN, O., AND MACALLUM, A. B.: On the blue color reaction of phosphotungstic acid (?) with uric acid and other substances. *J. Biol. Chem.*, 1912, **11**, 265.

15. FOLIN, O., AND MARENZI, A. D.: The preparation of uric acid reagent completely free from phenol reagent. *J. Biol. Chem.*, 1929, **83**, 109.

16. FOLIN, O., AND SHAFFER, P. A.: Über die quantitative Bestimmung der Harnsäure im Harn. *Z. physiol. Chem.*, 1901, **32**, 552.
    Eine Vereinfachung der Hopkins' schen Methode zur Bestimmung der Harnsäure im Harn. *Z. physiol. Chem.*, 1897, **24**, 224.

17. FOLIN, O., AND TRIMBLE, H.: A system of blood analysis. Supplement V. Improvements in the quality and method of preparing the uric acid reagent. *J. Biol. Chem.*, 1924, **60**, 473.

18. FOLIN, O., AND WU, H.: A system of blood analysis. *J. Biol. Chem.*, 1919, **38**, 81.

19. FRABOT, C.: Colour reaction for tungsten. *J. Chem. Soc.*, 1904, **86**, ii, 844; *Ann. Chim. anal.*, 1904, **9**, 371.

20. HADEN, R. L.: A modification of the Folin-Wu method for making protein-free blood filtrates. *J. Biol. Chem.*, 1923, **56**, 469.

21. HARPUDER, K.: Quantitative Bestimmung der Harnsäure im Blutserum. *Klin. Woch.*, 1923, **2**, 209.

22. HOPKINS, F. G.: On the estimation of uric acid in urine; a new process by means of saturation with ammonium chloride. *Proc. Roy. Soc. London*, 1892–3, **52**, 93.

23. HUNTER, G., AND EAGLES, B. A.: The isolation from blood of a hitherto unknown substance, and its bearing on present methods for the estimation of uric acid. *J. Biol. Chem.*, 1925, **65**, 623.

24. JACKSON, H., JR., AND PALMER, W. W.: A modification of Folin's colorimetric method for the determination of uric acid. *J. Biol. Chem.*, 1922, **50**, 89.
    A note on the determination of uric acid. *J. Biol. Chem.*, 1922, **53**, 373.

25. MORRIS, J. L.: A new salt of uric acid and its application to the analysis of uric acid and phenol. *J. Biol. Chem.*, 1916, **25**, 205.

26. MORRIS, J. L.: New titration method for the determination of uric acid in urine. *J. Biol. Chem.*, 1919, **37**, 231.

27. Morris, J. L., and MacLeod, A. G.: Colorimetric determination of uric acid. Estimation of 0.03 to 0.05 mg. quantities by a new method. *J. Biol. Chem.*, 1922, **50**, 55.
28. Pucher, G. W.: Studies on uric acid. I. Estimation of the variables in the Folin and Wu uric acid method. *J. Biol. Chem.*, 1922, **52**, 317.
II. A modification of the Folin and Wu uric acid method. *J. Biol. Chem.*, 1922, **52**, 329.
29. Rogers, H.: Exposure to light as a source of error in estimating uric acid by the Folin and Wu method. *J. Biol. Chem.*, 1923, **55**, 325.
30. Salkowski, E., and Ludwig, E.: in Tierfelder's Handbuch der physiologisch-und pathologisch-chemischen Analyze., p. 709, Berlin, 1924.
31. Wörner, E.: Ein einfaches Verfahren zur Bestimmung der Harnsäure auf Grund der Fällung als Ammonurat. *Z. physiol. Chem.*, 1900, **29**, 71.

CHAPTER XV

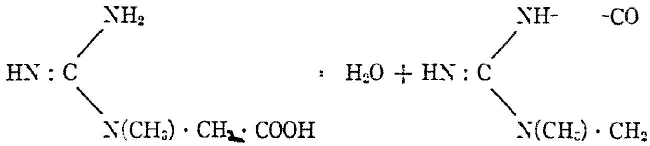
Creatine and Creatinine

PRINCIPLES OF METHODS AND APPLICABILITY TO URINE AND BLOOD

Principles

At present the only method for quantitative determination of creatinine is the colorimetric one based on the fact, discovered in 1886 by Jaffe (27), that this substance when treated at room temperature with picric acid in strongly alkaline solution forms an intense red color. Greenwald (21) has studied the nature of the colored product but without determining definitely its nature.

Creatine is determined indirectly by heating its solution with strong acid, which causes it to give off a molecule of water and change quantitatively into creatinine.



creatine. creatinine.

The creatinine thus formed is determined by the Jaffe reaction.

Application to urine

The quantitative application of the Jaffe reaction was accomplished in 1904 by Folin (11) who used it to determine both creatine and creatinine in urine. For this purpose the reaction has proved satisfactory, and the amounts of creatinine determined colorimetrically agree with those isolated from urine (29). There appear to be no other urinary constituents which to a measurable extent either give the reaction or affect the color produced with creatinine, except the rather rarely occurring acetone and acetoacetic acid. Acetone is said to diminish the color and acetoacetic acid to increase it (7). Both are readily removed by acidifying and boiling urine for a few minutes. In Folin's final method (13) their effect is claimed to be eliminated by the use of less alkali than in the original.

Conditions for heating with acid in order to change creatine into creati-

nine have been varied by different authors. Folin (11) originally mixed the urine with an equal volume of 1 N hydrochloric acid and heated on the water bath for three hours. F. G. Benedict and V. C. Myers (6) shortened the heating period to fifteen minutes by heating at 117° in an autoclave. The superheating increases the tendency to formation of dark products in the urine, however, and Hahn and Barkan (24) preferred to go to the other extreme and heat for twenty-four hours at 60° to 65°. S. R. Benedict (3) combined the speed of the autoclave method with freedom from darkening by boiling down to dryness the mixture of urine and acid in the presence of granulated lead, which prevented the formation of dark substances, so long as glucose was not present. All the above procedures used a mixture of urine with an equal volume of 1 N hydrochloric acid. Folin's final improvement (13) was to use 20 volumes of saturated picric acid in place of the HCl, and accomplish the conversion by boiling for an hour. Glucose up to 5 per cent does not interfere. The same mixture can be autoclaved for twenty minutes at 115° to 120°, but glucose interferes. All the above variations for urinary creatine were matters of convenience: they gave the same results, except in the presence of glucose.

Application to blood

When alkaline picrate is mixed with blood filtrate a red color is obtained. We have no idea at present what the chromogenic substance is, but it can not be all creatinine. The color reaction as a measure of blood creatinine was first used by Folin and Denis (15) in 1912 in experiments on animals treated with creatinine or creatine. On 1914 Folin (14) proposed a method utilizing the reaction for determination of naturally occurring creatinine in blood, and later with Wu (17) incorporated it into their well known system of blood analysis on tungstic acid filtrates.

By various authors the conditions influencing the color formation were studied and modifications of the method were proposed (8, 20, 23, 24, 26, 28, 29, 30), without any proof by isolation and identification that creatinine or creatine really exists in blood. The first doubt was perhaps caused by Hunter and Campbell (25) who found that whole blood filtrates mixed with alkaline picrate solutions continued to increase in color intensity for a half-hour or more, while a maximum with pure creatinine was reached in ten minutes. The rate of color formation in *plasma* filtrates was about normal for creatinine. Greenwald and McGuire (23) were unable to isolate any creatinine from blood. Behre and Benedict (1) in 1922 finally showed in several ways that but a small part, if any, of the color ordinarily formed with alkaline picrate in blood filtrates could be attributed to creatinine,

even when the chromogenic substance was greatly increased by renal retention. They confirmed Greenwald and McGuire (23) in finding that kaolin quantitatively adsorbs creatinine in amounts such as, from the color reaction, would be estimated to be present in blood filtrates. But kaolin did not remove any of the chromogenic substance from these filtrates. Also animal charcoal, which quantitatively adsorbed creatinine, either in pure solution or added to blood filtrates, failed to adsorb the chromogenic substance. Heating with 10 per cent NaOH was found to destroy the power of creatinine to deepen the color of picrate solution, but it did not so affect the power of blood filtrates. Methods of isolation which yielded creatinine when applied to solutions of that substance yielded none when applied to blood. Behre and Benedict decided that there might be some creatine present in blood, but practically no creatinine, and that the creatinine present in the urine is therefore presumably formed in the kidneys from some precursor.

Gaebler (18, 19) found that the chromogenic substance resembled creatinine in its precipitability by a mixture of potassium salts and picric acid, but not in other properties.

Despite our complete ignorance of the nature of the substances which cause the Jaffe reaction in blood filtrates, blood "creatinine" determinations by the method of Folin and Wu continue to be carried out in nearly all clinics where renal function is studied, including those of the authors. The justification for this practice is that clinical observation has shown that in nephritis an increase in the amount of chromogenic substance in the blood is of grave prognostic significance. It is regrettable only that this unknown substance or mixture of substances continues to be called "creatinine" in laboratory reports, and probably to be considered as creatinine by most physicians to whom such reports are rendered.

Concerning the "creatine" in the blood there is also uncertainty (18, 19) as to what part of the material estimated as such is really the substance. There appears not to be the excuse for its clinical determination that there is for that of "creatinine." We shall give the Folin-Wu method for blood creatine, merely because the analysis may at times be called for, and there is a possibility that the significance of the material determined may be clarified by future work.

REAGENTS FOR URINE AND BLOOD CREATININE DETERMINATIONS

Saturated solution of purified picric acid. Such a solution contains 12 grams of dry picric acid per liter. Picric acid as sold contains about 20 per cent of water to reduce the liability to explosion. Hence about 15

grams of the moist commercial product are taken to make a liter of the saturated solution.

The picric acid must pass the following test of Folin and Doisy (16). To 10 cc. of the saturated picric acid solution add 5 cc. of 10 per cent sodium hydroxide and let the mixture stand fifteen minutes. The color of the alkaline picrate solution thus made must not be more than twice as deep as the color of the saturated picric acid solution. If a deep color does form in alkaline solution impurities are present which form sufficient color to interfere with creatinine determinations (28). Such picric acid can be purified by methods described below.

Alkaline picrate solution. Mix 5 volumes of saturated picric acid solution with 1 volume of 10 per cent sodium hydroxide. The mixture of picric acid and alkali solutions should be made fresh on the day used.

A 10 per cent solution of sodium hydroxide.

Stock standard 0.1 per cent solution of creatinine. Ordinarily this solution is made from the easily purified creatinine zinc chloride. Of this 1.602 grams are dissolved and made up to 1 liter in 0.1 N hydrochloric acid solution. Each cubic centimeter of the resultant solution contains 1 mg. of creatinine.

Dilute standard creatinine solution for blood analyses. To 6 cc. of the above 0.1 per cent creatinine solution in a 1 liter flask add 10 cc. of 0.1 N hydrochloric acid and dilute to the mark with water. One cubic centimeter of this solution contains 0.006 mg. of creatinine.

Purification of picric acid. Benedict (5)

1. *Crystallization from glacial acetic acid.* This method is suited to small amounts. One hundred grams of the moist commercial picric acid are dried in air at 80° to 90°, or in a vacuum desiccator, and then dissolved in 150 cc. of glacial acetic acid, with the aid of heat which is continued until the mixture boils. The hot solution is poured on a fluted filter in a dry funnel which has been heated previously, and the filtrate is collected in a dry beaker. The beaker, covered with a watch glass, is allowed to stand over night at room temperature. If, at the end of this time, crystallization has not begun, it is initiated by vigorous stirring or by seeding with a crystal of picric acid. After two hours, or when crystallization is complete, the mixture is filtered with the aid of suction through a hardened filter and washed with about 35 cc. of cold glacial acetic acid. The precipitate is sucked as free from acid as possible and then dried at 80° to 90°, with occasional stirring, until it no longer smells of glacial acetic acid.

2. *Crystallization as sodium picrate.* This is suited to large amounts. It is essentially the method of Folin and Doisy (16) except that sodium carbonate is substituted for sodium hydroxide. Benedict states that the hydroxide causes slow decomposition of the picric acid, but the carbonate does not affect it. By the above Folin-Doisy test the picric acid obtained is slightly better than that from glacial acetic acid.

In a large porcelain enamelled pail without defects in the enamel 6 liters of water are heated to boiling. To this are added 250 grams of anhydrous sodium carbonate. When this has dissolved 500 grams of moist technical picric acid are added gradually. Before all the picric acid has dissolved the pail is removed from the flame and the contents are stirred

until solution is complete. After the solution has stood for a few minutes the fluid is decanted from the dirt which has settled to the bottom of the pail (filtration is usually unnecessary). It is allowed to stand overnight at room temperature. At the end of this time the crystallized sodium picrate is collected on a large Buchner funnel with the aid of suction which is continued until the precipitate is dry. It is then washed with 2 liters of 10 per cent sodium hydroxide and again sucked dry. The suction is now turned off, 500 cc. of diluted hydrochloric acid (25 volumes per cent of concentrated HCl) is poured on the filter, and the mixture is stirred thoroughly with a porcelain spatula. The picrate is converted by the HCl into crystals of free picric acid. The fluid is then sucked into a receiving flask. The picric acid on the funnel is washed with water three times, after which the precipitate is sucked dry. The material is removed from the filter, dried at about 90° and powdered.

Both methods should give picric acid which, when tested by the above described method of Folin and Doisy, proves satisfactory for creatinine determinations. The final product should be preserved in brown, glass-stoppered bottles.

Benedict's method for the preparation of pure creatinine-zinc chloride from urine (2)

To 10 liters of undecomposed urine in a large precipitating jar add a hot solution containing 180 grams of picric acid in 450 cc. of boiling alcohol. Allow the solution to stand over night and syphon off the supernatant fluid. Pour the residue on a large Buchner funnel, drain with suction, wash it once or twice with cold, saturated picric acid solution and suck dry. To every 100 grams of the picrate add about 60 cc. of concentrated hydrochloric acid, enough to form a moderately thin paste, and stir the mixture thoroughly with the pestle for from three to five minutes. Filter it with suction on a hardened filter paper and wash the precipitate twice with enough water to cover it, sucking it as nearly dry as possible each time. Transfer the filtrate to a large flask and neutralize it with an excess of "solid, heavy" magnesium oxide, using litmus paper as an indicator. Add the oxide in small portions and cool the flask under running water after each addition. Filter the mixture with suction and wash the residue twice with water. Immediately add a few cubic centimeters of glacial acetic acid to the filtrate to make it strongly acid and dilute the solution with about 4 volumes of 95 per cent alcohol. After fifteen minutes filter off the slight precipitate. To the final filtrate add 30 to 40 cc. of 30 per cent zinc chloride. Stir the mixture and let it stand over night in a cool place. Pour off the supernatant liquid. Collect the creatinine zinc chloride on a Buchner funnel. Wash it once with water, then thoroughly with 50 per cent alcohol, finally with 95 per cent alcohol, and dry. A nearly white, light, crystalline powder, creatinine-zinc chloride, should be obtained.

This should be recrystallized according to the method of Folin (12) as follows: Dissolve the crude creatine-zinc chloride in about 10 times its weight of boiling 25 per cent acetic acid. To the mixture add 2 volumes of 95 per cent alcohol. On cooling, pure creatinine-zinc chloride separates almost quantitatively.

Edgar's (9, 10) method for the preparation of creatinine-zinc chloride from creatine

Grind commercial creatine in a mortar with an equal weight of anhydrous zinc chloride. Heat the mixture in a beaker, dish or casserole over a small flame or a sand bath, stirring it constantly. At a temperature of 120° to 130°C. the material becomes a viscous mass from which bubbles of water vapor are given off. Within a few minutes it suddenly solidifies to a perfectly dry residue consisting of creatinine-zinc chloride, containing the excess zinc chloride. The latter can be dissolved out by leaching the mass with a little cold water, followed by 50 per cent and then 95 per cent alcohol, as described above.

The crude creatinine-zinc chloride is then recrystallized by the method of Folin described above.

Greenwald (22) has suggested ammonium creatinine picrate as a suitable salt for the isolation and purification of creatinine.

DETERMINATION OF CREATININE IN URINE. FOLIN (13)

Place 2 cc. of urine in a 100-cc. flask. In another similar flask place 1 cc. of the stock standard creatinine solution, containing 1 mg. of creatinine per cubic centimeter. To each flask add 20 cc. of the picric acid solution and, from a burette, 1.5 cc. of 10 per cent sodium hydroxide. Let the solutions stand ten minutes. At the end of that time dilute to the mark with water, mix and compare unknown with standard in the colorimeter. If the readings of standard and unknown differ by more than 50 per cent, the determination must be repeated with a more suitable amount of urine.

Calculation

$$\frac{S}{U\,V} = \text{grams of creatinine in a liter of urine.}$$

$$\frac{0.372\,S}{U\,V} = \text{grams of creatinine nitrogen in a liter of urine.}$$

S = reading of standard, U = reading of unknown and V = the cc. of urine taken for analysis.

SUM OF CREATININE AND CREATINE IN URINE: "TOTAL CREATININE"

Creatine present is changed by heating with acid to creatinine and the total creatinine present is determined. Subtraction of the preformed creatinine, determined separately as above described, yields by difference the creatine.

Three procedures for carrying out the heating with acid are described.

1. The Folin open flask method is the only one of the three that can be used if the urine contains glucose. Up to 5 per cent of glucose does not interfere.

2. The Folin autoclave technique is most convenient when many analyses are done together.

3. The Benedict boiling down procedure is quickest and simplest for a few analyses.

1. Folin's open flask method (13)

To 1 cc. of urine in a 300-cc. flask add 20 cc. of saturated picric acid solution. Weigh the flask and its contents to within 0.1 gram, and add

about 150 cc. of water. Boil gently for forty-five minutes and then more rapidly until the volume of fluid in the flask is about 20 cc. Cool. Place the flask on the scales again and add enough water to restore the weight of the original 21 cc. of solution. To 1 cc. of the stock standard creatinine in another flask add 20 cc. of saturated picric acid solution. To both standard and unknown are now added 1.5 cc. of 10 per cent sodium hydroxide. After they have stood ten minutes compare them in the colorimeter.

2. Folin's autoclave method (13)

To 1 cc. of urine in a 100-cc. volumetric flask add 20 cc. of the saturated picric solution. Cover the mouth of the flask with tin-foil and heat it in an autoclave at 115° to 120°C. for twenty minutes. Cool. Place 1 cc. of the stock standard creatinine solution in another similar flask with 20 cc. of saturated picric acid. To each flask add 1.5 cc. of 10 per cent sodium hydroxide solution. After they have stood ten minutes, dilute to the mark, mix and compare standard and unknown in the colorimeter.

3. Benedict's boiling down method (3)

To 2 cc. of urine in a medium sized test tube add 2 cc. of 10 per cent (by volume) hydrochloric acid and a little powdered metallic lead. Evaporate the contents of the tube carefully nearly to dryness by boiling over a free flame, and then wash them with as little water as possible through a very small cotton or glass wool filter into a 100-cc. volumetric flask to remove the metallic lead. In another 100-cc. volumetric flask place 1 cc. of the stock standard creatinine. Into each flask introduce 20 cc. of the saturated picric acid. To the unknown add 2 cc. and to the standard 1.5 cc. of 10 per cent sodium hydroxide. After they have stood ten minutes dilute to the mark, mix and compare standard and unknown in the colorimeter.

Calculation

$\frac{S}{UV}$ = grams of "total creatinine" from both creatine and preformed creatinine in a liter of urine

$$\frac{0.372\ S}{UV} = \text{grams of "total creatinine" nitrogen in a liter of urine.}$$

Where S and U = readings of standard and unknown respectively and V = cc. of urine taken for analysis.

("Total creatinine N") − (preformed creatinine N) = creatine N.

APPARENT "CREATININE" IN BLOOD. FOLIN AND WU (17)

Procedure

Place 10 cc. of Folin and Wu blood filtrate (see precipitation of proteins, p. 65), in a small flask or test tube and 5 cc. of the dilute standard creatinine solution together with 15 cc. of water in another receptable. To the blood filtrate add 5 cc. and to the standard solution 10 cc. of the freshly prepared alkaline picrate. After eight or ten minutes compare the two in the colorimeter. Not more than fifteen minutes should elapse between the addition of the alkaline picrate and the completion of the color comparison.

It is essential that the depth of color in standard and unknown be approximately the same. To ensure this it is well to prepare more than one standard with each test. The standard described above is useful only for normal "creatinine" values of 1 to 2 mg. per 100 cc. For higher "creatinine" values one uses 10 cc. of standard plus 10 cc. of water, or 15 cc. of standard plus 5 cc. of water, or 20 cc. of standard. If a high "creatinine" should be encountered unexpectedly without an appropriate standard ready, the determination can be saved by diluting the unknown with the akaline picrate solution (first diluted with two volumes of water) until the color of the unknown approximates that of the nearest standard.

Calculation

$$\frac{0.3\ C\ S}{R} = \text{milligrams of "creatinine" in 100 cc. of blood.}$$

Where S and R represent the readings of standard and unknown respectively, and C the cubic centimeters of standard creatinine solution measured into the standard solution. ($C = 5$ for normal blood.)

As stated above, it is uncertain what the substance thus calculated as "creatinine" is, except that it is not creatinine.

APPARENT "TOTAL CREATININE" IN BLOOD. FOLIN AND WU (17)

Transfer 5 cc. of the Folin and Wu tungstic acid filtrate (see precipitation of proteins, p. 65), to a test tube graduated to 25 cc. Add 1 cc. of 1.0 N hydrochloric acid. Cover the mouth of the test tube with tinfoil and heat in an autoclave to 130°C. for twenty minutes or to 155°C. for ten minutes. Cool. Place 10 cc. of the diluted creatinine standard solution described above in a 50 cc. volumetric flask and add to it 2 cc. of 1.0 N hydrochloric acid. When the unknown has cooled add to it

5 cc. of the alkaline picrate solution and to the standard solution add 10 cc. of the same solution. Allow both to stand 5 to 10 minutes. Then dilute the unknown to 25 cc. and the standard to 50 cc. and compare them in the colorimeter.

Calculation

$$\frac{6S}{U} = \text{milligrams of "total creatinine" in 100 cc. of blood.}$$

Where S and U represent the readings of standard and unknown respectively, and C the cubic centimeters of standard solution taken for comparison.

It has been customary to consider that the figures thus calculated represent the sum of creatinine and creatine. In the introductory discussion it has been shown that no one knows what they represent.

BIBLIOGRAPHY

1. Behre, J. A., and Benedict, S. R.: Studies in creatine and creatinine metabolism. IV. On the question of the occurrence of creatinine and creatine in blood. *J. Biol. Chem.*, 1922, **52**, 11.
2. Benedict, S. R.: Studies in creatine and creatinine metabolism. I. The preparation of creatine and creatinine from urine. *J. Biol. Chem.*, 1914, **18**, 183.
3. Benedict, S. R.: Studies in creatine and creatinine metabolism. II. The estimation of creatine. *J. Biol. Chem.*, 1914, **18**, 191.
4. Benedict, S. R.: A method for the purification of picric acid for creatinine determinations. *J. Biol. Chem.*, 1922, **54**, 239.
5. Benedict, S. R.: A note on the purification of picric acid for creatinine determination. *J. Biol. Chem.*, 1929, **82**, 1.
6. Benedict, F. G., and Myers, V. C.: The determination of creatine and creatinine. *Am. J. Physiol.*, 1907, **18**, 397.
7. Blau, N. P.: The estimation of creatinine in the presence of acetone and diacetic acid. *J. Biol. Chem.*, 1921, **48**, 105.
8. Denis, W.: The determination of creatinine and creatine in blood. *J. Biol. Chem.*, 1918, **35**, 513.
9. Edgar, G.: Preparation and comparison of standards for the estimation of creatine and creatinine. *J. Biol. Chem.*, 1923, **56**, 1.
10. Edgar, G., and Hinegardner, W. S.: The preparation of creatinine from creatine. *J. Biol. Chem.*, 1923, **56**, 881.
11. Folin, O.: Beitrag zur Chemie des Kreatinins und Kreatins in Harne. *Z. Physiol. Chem.*, 1904, **41**, 223.
    Approximately complete analyses of thirty "normal" urines. *Am. J. Physiol.*, 1905, **13**, 45.
12. Folin, O.: On the preparation of creatine, creatinine, and standard creatinine solutions. *J. Biol. Chem.*, 1914, **17**, 463.
13. Folin, O.: On the determination of creatinine and creatine in urine. *J. Biol. Chem.*, 1914, **17**, 469.

14. Folin, O.: On the determination of creatinine and creatine in blood, milk, and tissues. *J. Biol. Chem.*, 1914, **17**, 475.
15. Folin, O., and Denis, W.: Protein metabolism from the standpoint of blood and tissue analysis. III. Further absorption experiments with especial reference to the behavior of creatine and creatinine and to the formation of urea. *J. Biol. Chem.*, 1912, **12**, 141.
VII. An interpretation of creatine and creatinine in relation to animal metabolism. *J. Biol. Chem.*, 1914, **17**, 493.
16. Folin, O., and Doisy, E. A.: Impure picric acid as a source of error in creatine and creatinine determination. *J. Biol. Chem.*, 1917, **28**, 349.
17. Folin, O., and Wu, H.: A system of blood analysis. *J. Biol. Chem.*, 1919, **38**, 81.
18. Gaebler, O. H.: Further studies on blood creatinine. *J. Biol. Chem.*, 1930, **89**, 451.
19. Gaebler, O. H., and Keltch, A. K.: On the nature of blood creatinine. *J. Biol. Chem.*, 1928, **76**, 337.
20. Gettler, A. O.: Factors involving the accuracy of creatinine determinations in human blood. *J. Biol. Chem.*, 1917, **29**, 47.
21. Greenwald, I.: The chemistry of Jaffe's reaction for creatinine. IV. A compound of creatinine, picric acid, and sodium hydroxide. *J. Biol. Chem.*, 1928, **77**, 539.
22. Greenwald, I.: A note on ammonium creatinine picrate and its possible use in the preparation of creatinine. *J. Biol. Chem.*, 1929, **81**, 73.
23. Greenwald, I., and McGuire, G.: The estimation of creatinine and of creatine in the blood. *J. Biol. Chem.*, 1918, **34**, 103.
24. Hahn, A., and Barkan, G.: Über die gegenseitige Umwandlung von Kreatin und Kreatinin. II. Mitteilung. *Z. Biol.*, 1920, **72**, 305.
25. Hunter, A., and Campbell, W. R.: The probable accuracy, in whole blood and plasma, of colorimetric determinations of creatinine and creatine. *J. Biol. Chem.*, 1917, **32**, 195.
26. Hunter, A., and Campbell, W. R.: A hitherto neglected factor affecting the determination of minute quantities of creatinine. *J. Biol. Chem.*, 1917, **28**, 335.
27. Jaffe, M.: Über den Niederschlag welchen Pikrinsäure in normalen Harn erzeugt, und über eine neue Reaction des Kreatinins. *Z. physiol. Chem.*, 1886, 10, 391.
28. McCrudden, F. H., and Sargent, C. S.: The occurrence and determination of creatine in the urine. *J. Biol. Chem.*, 1916, **24**, 423.
The influence of the color from the sodium picrate in the determination of creatinine in blood and urine. *J. Biol. Chem.*, 1916, **26**, 527.
29. Morris, J. L.: Determination of creatine and creatinine. The occurrence of creatine. *J. Biol. Chem.*, 1915, **21**, 201.
30. Wilson, D. W., and Plass, E. K.: Creatine and creatinine in whole blood and plasma. *J. Biol. Chem.*, 1917, **29**, 413.

CHAPTER XVI

Lactic Acid

PRINCIPLES OF METHODS

The classical method for determination of lactic acid involves its separation, usually by ether extraction combined with other means, from interfering substances, its conversion into zinc lactate by boiling with zinc oxide or carbonate, and the weighing of the lactate. The purity of the latter can then be determined by analysis. This method was used in the famous studies of lactic acid formation in muscle by Fletcher and Hopkins (8) and was applied to blood by Wolf (28). Because the purity of the salt can be proven, this procedure still remains a standard method. It is, however, time consuming because of the prolonged extraction required to remove lactic acid from other biological material, and not suited to determination of small amounts.

In consequence other methods have been devised which are more rapid and suited to micro analyses, although not capable of the same rigid analytical proof of specificity as the zinc lactate procedure. Such methods thus far developed depend upon decomposition of the lactic acid and estimation of one of the products, acetaldehyde, CO_2, or CO.

When heated with potassium permanganate or manganese dioxide suspension, lactic acid is oxidized to acetaldehyde and carbon dioxide:

$$CH_3 \cdot CH(OH) \cdot COOH + O = CH_3 \cdot CHO + CO_2 + H_2O.$$

Boas (2) and Jerusalem (14) distilled the aldehyde into alkaline 0.1 N iodine and estimated the aldehyde from the amount of iodine reduced. Von Fürth and Charnass (12), finding the direct iodimetric titration inaccurate, distilled into bisulfite and used the Ripper (22) titration of the amount of $NaHSO_3$ bound by the aldehyde to determine the latter.

By Baumberger and Field (1) the CO_2 formed by the permanganate oxidation, carried out in blood filtrate at room temperature in the Van Slyke-Neill gas apparatus, was measured in a gasometric determination of lactic acid. Hastings and Avery developed the same procedure independently and applied it to the Folin-Wu blood filtrate (personal communication). The determination of CO_2 has a theoretical advantage over determination of the acetaldehyde. If excess permanganate is allowed to act on the alde-

hyde it is further oxidized and the result of the analysis is too low. Addition of permanganate in all modifications of the von Fürth-Charnass method is cautious in order to avoid error from this source. The CO_2 formed, however, can not be further oxidized.

Another decomposition reaction is undergone when lactic acid is heated with strong sulfuric acid at 100°. It yields again acetaldehyde, but the other product is CO instead of CO_2 (Denigès, (6)).

$$CH_3 \cdot CH(OH) \cdot COOH = CH_3 \cdot CHO + CO + H_2O.$$

As nearly as one can judge from available data, determination of the aldehyde yielded by this reaction gives nearly true results for lactic acid in the ether extract of urine (24), and determination of the CO gas gives good results with blood filtrates (4, 20, 23). Urine extract contains, however, substances other than lactic acid which yield CO (19), and blood filtrate contains other substances which yield acetaldehyde (23), the results in both these cases being definitely too high.

For determination of the acetaldehyde yielded by this reaction both the Clausen titration and several colorimetric methods have been used. Ryffel (24) used the color reaction with Schiff's reagent (rosaniline hydrochloride decolorized with sulfur dioxide), Harrop (13) the color formed with guiacol, and Mendel and Goldschneider (21) the color formed with veratrol. The last mentioned reagent has the practical advantage that it can be used in the sulfuric acid mixture, so that the aldehyde need not be driven into another vessel for estimation.

Choice of Methods

For blood we shall describe three methods:

1. The most recent improvement of the von Fürth-Charnass iodometric procedure by Friedemann, Cotonio, and Shaffer (10) and Friedemann and Kendall (11). This method with its iodometric titration is the result of years of development begun by Clausen (4) and carried on by Shaffer's collaborators (5, 9, 10, 11) and must be at present accepted as the most thoroughly applied and tested procedure available. The entire determination, including oxidation, collection of aldehyde, and titration, can be carried through in fifteen or twenty minutes.

2. The direct colorimetric method of Mendel and Goldschneider (21). This method has not attained the general application of the older von Fürth-Charnass, but its accuracy has been confirmed by Vas and Lang (26). The veratrol reagent is applied directly to blood filtrate. It obviates the aeration involved in the preceding method, and provides a colorimetric alterna-

tive. For these reasons it is included. Its behavior in general clinical application remains to be demonstrated, and those who use it at present must assume the responsibility of controlling it.

3. The Avery and Hastings gasometric method. This is the simplest procedure. Like the colorimetric method, it is new and has not yet received general clinical application. It appears certain, however, that it will suffice to measure pathological increases of blood lactic acid. It is described on page 427 in the chapter on gasometric methods.

For urine we describe only the Friedemann-Cotonio-Shaffer iodometric method. The application of the other two procedures to urine has not been made.

In specificity these methods are in a state much like that of the blood sugar methods. Each method has been found to determine 95 per cent or more of lactic acid added to blood or urine, but it has not yet been demonstrated whether the preexisting material determined in these fluids by any of the methods is exclusively lactic acid. It is probable, as in the case of blood glucose methods, that some of the material determined in normal blood and urine is not lactic acid, but that pathologically significant increases are shown with sufficient accuracy by any one of the three procedures. Clausen (4) and Friedmann (11) have shown that other substances which can occur in blood filtrates and urine give off acetaldehyde or volatile products which titrate as such under the conditions of the permanganate-iodometric method. Long (18) compared the Clausen (4) form of this method with the color given by blood filtrates with Hopkins' (8) thiophene reaction. He obtained 25 per cent more apparent lactic acid by the permanganate-iodometric method than by the thiophene colorimetric estimation, and concluded that about 75 per cent of the substance determined in normal blood by the Clausen procedure was lactic acid and 25 per cent other substance. The later Friedeman-Cotonio-Shaffer (10) form of the permanganate-iodometric method was found, however, by Ronzoni and Wallen-Lawrence (23) to give in blood filtrates consistently the same results as estimation of the carbon monoxide yielded by the sulfuric acid reaction. The substitution of phosphoric for sulfuric acid in the oxidation mixture by Friedemann and Kendall (11) was later found to give in human blood lactic acid values still lower by 1 or 2 mg. per 100 cc. (0.1 or 0.2 millimoles per liter). It appears probable that the lactic acid values given by the present form of the permanganate-iodometric method in blood closely approximate the actual figures. Tests of the specificity of the colorimetric procedures for blood are lacking.

In urine Friedemann and Kendall (11) have found that the ether extract when treated with permanganate by their method yields only about one-third as much volatile substance titrating as aldehyde as is yielded by the whole urine. If instead of ether extraction the urine was treated with copper sulfate and calcium hydroxide (procedure used in Van Slyke acetone determination to remove glucose, see p. 626) the filtrate yielded only about as much aldehyde as the ether extract. When both methods for removal of interfering substances were applied together still lower results were obtained. For example, untreated urine gave aldehyde equivalent to 26 mg. of lactic acid per 100 cc., copper-lime filtrate 10 mg., ether extract 8 mg. ether extract cleared by copper-lime 5.5 mg. No standard procedure for preparation of urine appears to have been settled upon as the result of systematic experiment.

PERMANGANATE IODOMETRIC METHOD FOR URINE AND BLOOD, MODIFIED VON FÜRTH-CHARNASS TECHNIQUE (12, 4, 5, 10, 11)

The permanganate oxidation discussed above is used, and the aldehyde is driven into bisulfite solution by a current of air and titrated by Clausen's (4) modification of Ripper's (22) method. The present technique is the result of so many detailed improvements in the von Fürth-Charnass method by various authors that it is impossible to give it the name of any one.

In the original Ripper aldehyde titration as used by von Fürth and Charnass the aldehyde was collected in an excess of standard bisulfite, with which it combines, molecule for molecule.

$$CH_3CHO + NaHSO_3 = CH_3CHO \cdot NaHSO_3.$$

The bisulfite compound does not react with iodine, while the unbound $NaHSO_3$ does react:

$$NaHSO_3 + 2I + H_2O = NaHSO_4 + 2\ HI.$$

In the Ripper method the excess $NaHSO_3$ is titrated with iodine. One cubic centimeter of 1 N iodine (1 millimole) titrates 0.5 millimole or 45 mg. of lactic acid. The disadvantage of the Ripper method is that it is a titration by difference. Clausen converted it into a direct titration by taking advantage of the fact that the $CH_3CHO \cdot NaHSO_3$ compound, although stable in acid or neutral solution, splits into its components when made alkaline. Consequently Clausen added iodine to the acid bisulfite-aldehyde solution until all the free sulfite was oxidized, then added alkali and performed a direct titration on the remaining sulfite, which had been bound to aldehyde. Clausen also introduced aeration in place of distillation for driving the aldehyde into the bisulfite, and applied the copper-lime precipitation of Van Slyke for preliminary removal of sugar and other interfering substances from blood filtrates.

Friedemann, Cotonio, and Shaffer (10) showed that the addition of manganous salts to the oxidation mixture greatly accelerated the formation of aldehyde, and, by reducing the Mn^{vii} to Mn^{iv}, decreased the danger of oxidation past the stage of aldehyde. They also introduced the principle of driving the current of air up through a cooled condenser, where some less volatile products are retained which, if they reached the bisulfite, would titrate as aldehyde. Friedemann and Kendall (11) substituted phosphoric acid for the sulfuric previously used in the oxidation mixture, and obtained a decrease in the amount of aldehyde yielded by substances other than lactic acid.

Except for the preliminary treatment to remove interfering substances, the method is the same for blood and urine.

Apparatus

The apparatus, which is shown in figure 79, can be constructed from ordinary stock glass-ware. It consists, essentially, of a boiling flask fitted to a reflux condenser through which an air current carries the volatile aldehyde into an absorbing tube or tower containing bisulfite. Two large test tubes in tandem may be substituted for the bead tower shown in the figure.

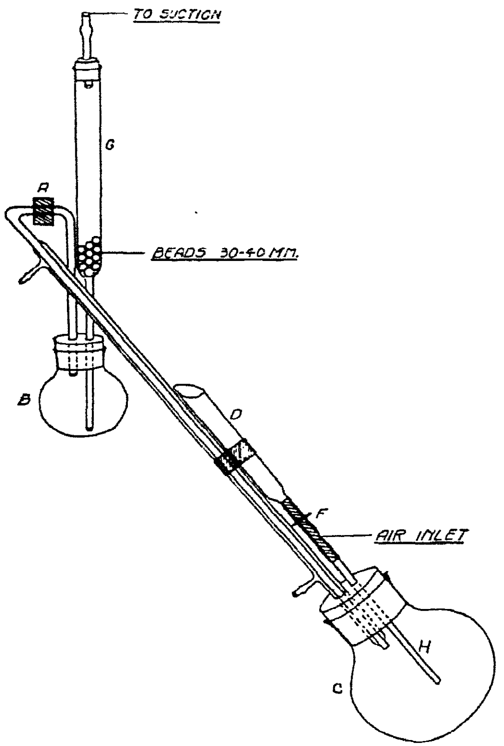


FIG. 79. Apparatus of Friedemann, Cotonio and Shaffer (8) as modified by West (27) for the determination of lactic acid.

The apparatus consists of the three units, boiling flask, condenser, and absorber, all of Pyrex glass. The boiling flask *C*, a wide mouth extraction flask of 250 cc. capacity, is fitted by a rubber stopper to a condenser unit made from a West type condenser. The condenser should have an inner tube of thin wall Pyrex, about 7 mm. inside diameter with 0.5 to 1-mm. space between the inner and outer tubes (water space) and a water column about 425 mm. long. A standard wall Pyrex tube (about 8 mm. outside diameter) is sealed to the condenser and bent for attachment to the absorber *B*. The absorber consists of a 150 cc. wide mouth extraction flask fitted with a tube *G*, containing a few beads as shown. *G* is conveniently made from a 25 by 200 mm. Pyrex test-tube, into the bottom of which a 7 by 140 mm. tube is sealed. *D*, the permanganate or manganese dioxide container, is prepared from a small Pyrex test-tube and attached by rubber tubing to the delivery tube, *H*. *F* is a screw clamp to control the flow of oxidizing agent into the flask. A small hole, cut in the rubber below *F*, serves as an air inlet. *D* is fastened to the condenser with friction tape. The apparatus is loosely supported in the split cork, *A*, by a clamp or by friction tape to a horizontal rod, and under the neck of the boiling flask or low on the condenser by a horizontal rod. The slope of the condenser, relative to the desk top, should be about 45 degrees. Mounting in this way insures freedom of movement in the vertical plane without strains. A micro burner is used to heat the boiling flask.

Several units may be compactly assembled in a row on parallel horizontal rods, one attached loosely at *A*, and the other simply passing under the lower part of the condensers.

The condenser units may be more simply made by fastening the outer jacket in place with rubber connections. The inner tube is extended beyond the jacket and bent to enter the absorber as explained above. Such condensers, while not quite so efficient as the all glass ones with the thin inner tubes, are entirely satisfactory.

Davenport and Cotonio (5) have described a variation of this apparatus which may be preferred by some. It is essential that the condenser be efficient and well cooled. The Hopkins type seems to be best suited for that purpose. The space between the inner and outer tubes should not exceed 3 mm. and the height of the condensing surface should be at least 25 cm. This insures condensation of all the water vapor, and is also said to hold back some interfering volatile organic products.

Reagents

Oxidizing solution, colloidal manganese dioxide. This can be prepared by any one of the three following methods (10).

1. Nine grams of glucose, dissolved in 1 liter of approximately 0.5 N NaOH, are added to 3 to 4 liters of water in which 53 grams of potassium permanganate have been dissolved. The mixture is warmed to 70° and kept at this temperature for fifteen minutes. If the color disappears during this procedure, saturated aqueous potassium permanganate is added until an excess remains. The solution is then cooled, filtered through a large Buchner funnel and washed well with water. The precipitate of MnO_2, granular at first, becomes finer and finer as alkali and salts are removed. To facilitate the removal of salts, the precipitate is thoroughly broken up in a small amount of water (an egg-beater is an effective aid) and again returned to the Buchner funnel and washed. The salt-free precipitate is finally broken up thoroughly in water, diluted to 2 to 4 liters and set aside to settle. After a few hours the coarser particles settle out. After twelve to twenty-four hours the upper two-thirds of the suspension, which is practically free from visible particles, is removed by suction. The remainder, containing coarser particles, is again suspended, agitated and allowed to settle. This may be repeated several times. The supernatant colloidal suspensions are united and adjusted to a strength of 0.05 or 0.1 N. The strength is determined by mixing a known amount with a solution of potassium iodide acidified with H_2SO_4, and titrating with standard sodium thiosulfate (see p. 32). One cubic centimeter of 0.1 N thiosulfate = 1 cc. 0.1 N colloidal MnO_2. Suspensions prepared in this manner are not entirely colloidal. However, the sediment which settles out is not granular and can be resuspended readily. The MnO_2 is precipitated by electrolytes. The yield by this method is not large; only 50 to 70 per cent of the theoretical quantity is obtained after 5 suspensions.

2. A fairly stable and satisfactory suspension is secured by allowing potassium permanganate to react with manganous sulfate. A concentrated solution of manganous sulfate, containing slightly more than 3 equivalents (7 grams of $MnSO_4 \cdot 4H_2O$) is run, with stirring, into a solution of potassium permanganate containing 2 equivalents (3 grams of $KMnO_4$). The granular precipitate of manganese dioxide is washed free from salts by the procedure described in 1, above.

3. A fairly satisfactory dilute suspension for use in the analysis of blood and tissue extracts or in samples which contain relatively small amounts of oxidizable material other than lactic acid, may be prepared by adding a dilute manganous sulfate solution drop by drop to a dilute (0.01 N) potassium permanganate solution until the color is almost discharged.

The stock solution of 0.1 N manganese dioxide suspension is diluted as needed to 0.01 N.

A 2 M solution of phosphoric acid, prepared by diluting 135 cc. of syrupy (85 per cent) acid to 1 liter.

A 10 per cent solution of manganous sulfate ($MnSO_4 \cdot 4H_2O$).

Finely powdered talcum.

A 1 per cent solution of sodium bisulfite.

Starch indicator, prepared as described on page 34.

A saturated solution of sodium bicarbonate.

A 0.1 N solution of iodine. The 0.1 N iodine is diluted each day to 0.01 or 0.002 N and must be protected from strong sunlight. (For the preparation of the iodine, thiosulfate and biiodate solutions, see chapter I.)

A 25 per cent solution of copper sulfate ($CuSO_4 \cdot 5\ H_2O$).

Dry calcium hydroxide.

Phosphate buffer of pH 7. Sixty-two cubic centimeters of 0.3 M Na_2HPO_4 mixed with 38 cc. of 0.3 M KH_2PO_4.

Preparation of urine

Either of the following two methods of preparation may be used.

1. ***Ether extraction.***

To separate lactic acid from interfering substances in urine Clausen (4) extracted the acid with ether. To remove phenols the urine is first brought to pH approximately 7, at which all the lactic acid is bound as alkali salt while all the weakly acid phenol remains free, and the phenols are removed by a preliminary extraction.

For the extraction the apparatus shown in figure 80 is used. ***K*** is a 300 cc. Kjeldahl flask connected with a Hopkins reflux condenser, H. ***F*** is a funnel made from a test tube. Its end should be slightly bent and its opening should be about 0.5 mm. in diameter. ***E***, the extraction tube, blown from a test tube, has a side opening, ***O***, for ether outflow, and a small bulb at its lower end. The narrow lower portion should contain about 2 cc. in a column about 10 cm. deep. A copper wire, ***W***, holds ***E*** and is carried between the cork and the flask. As ether condenses and enters ***F***, it gradually displaces the fluid in the stem, which should be long enough to give a sufficient head of pressure to carry a rapid stream of ether in drops through the liquid in ***E*** until there is a steady outflow through ***O***. If at first bubbles of vapor in the stem of ***F*** prevent the flow, it is only necessary momentarily to make air pressure at the side tube of the Hopkins condenser.

As a preliminary to ether extraction it is necessary to remove al-

bumin, if present. To 5 cc. of urine add 0.5 cc. of 10 per cent sodium tungstate and 0.5 cc. of 1 N sulfuric acid. After precipitation the urine is filtered.

To remove phenols by preliminary extraction, 2 cc. of the albumin-free urine filtrate are placed in the extraction tube, a few drops of the

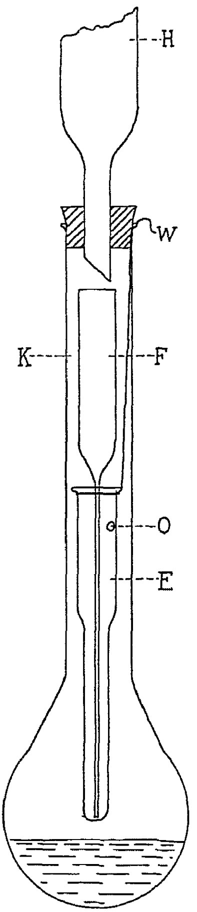


FIG. 80. Apparatus of Clausen (4) for the extraction of urine with ether as a preliminary to the determination of lactic acid.

phosphate buffer solution of pH 7.0 are added, and the mixture is extracted with ether for fifteen minutes.

1.3 grams of ammonium sulfate and a few drops of concentrated sul-

furic acid are now added to the treated urine in the extraction tube. A fresh flask containing 50 cc. of ether is then applied and the urine is reextracted for thirty minutes. The flask should be heated in a water bath at 60° to 70°C.

To the ether are now added about 10 cc. of water, 1 drop of 0.5 per cent phenolphthalein, and enough 0.1 N sodium hydroxide to make it just alkaline. The ether is then removed by distillation. The residue is transferred quantitatively to a small beaker and evaporated nearly, but not quite, to dryness on a water bath.

The residue is then washed with a small quantity of water into the flask *C* of figure 79, and the determination of lactic acid is carried out by the procedure described below.

2. ***Copper-line treatment.*** The urine is treated with copper sulfate and calcium hydroxide as described on page 615 in Van Slyke's method for removal of glucose. The treatment also removes other interfering substances (11) almost as completely as the ether extraction, and is considerably simpler than the ether extraction. Twenty cubic centimeters of the the filtrate, equivalent to 2 cc. of urine, are transferred to flask *C* of figure 79 and used for the lactic acid determination.

Preparation of blood

If the proteins can not be precipitated within five minutes after the blood is drawn, the blood should be drawn into a receiver containing enough NaF or NH_4F to make a 1 per cent solution, in order to prevent lactic acid formation from glycolysis (3, 7, 15, 16).

The proteins are removed from blood or serum by tungstic acid according to Folin and Wu (see page 65). To 10 cc. or more of protein-free filtrate add one-tenth its volume of 25 per cent copper sulfate and enough dry calcium hydroxide to make the solution alkaline. The mixture is shaken at intervals for one-half hour and then centrifuged. Five cubic centimeters of the supernatant fluid from the lime and copper treatment, equivalent to 5/11 cc. of blood or serum, are transferred to the 300-cc. round flask of figure 79, and analyzed as described below.

Oxidation

The prepared filtrate or extract containing preferably 0.5 to 2.0 mg. of lactic acid is transferred to the 300-cc. round flask *C* of figure 79, and to it are added 4 or 5 cc. of 2 M phosphoric acid, 10 cc. of the 10 per cent manganous sulfate, a pinch of talcum powder to promote even boiling,

and enough water to bring the volume of solution to 80 or 100 cc. The flask is then connected to the condenser, and the condenser is connected to an empty receiving flask (without the bead tower). To remove volatile products, such as acetone and acetoacetic acid, which could be titrated as aldehyde, the liquid in flask *C* is heated to boiling with a micro burner while a current of air is drawn through it and the empty flask for five minutes. (This preliminary heating and aeration may be omitted if no interfering volatile products are present.) The flame is then removed and aeration is stopped while a fresh receiving flask *B* and the bead tower shown in figure 79 are put in place. Into flask *B* are put 5 to 10 cc. of the sodium bisulfite solution and enough water to cover the beads in the tower when the fluid is drawn up into the latter by suction. Heating and aeration are then resumed, and 0.01 N MnO_2 suspension is dropped in from the funnel tube at a rate of not more than 1 or 2 drops per second, until an excess has accumulated in the flask, giving the mixture a pink color. An actual excess of MnO_2 should be present for at least ten minutes. If the solution becomes decolorized more MnO_2 must be added. The addition of the oxidizing reagent should be continued until there is a no further tendency for the pink color to fade and until manganese dioxide separates out. This requires up to ten minutes. Aeration is carried out for a total of twenty minutes, time being counted from the moment the oxidation is started. The flame is then removed, the air current stopped, receiving flask *B* lowered, and the tower rinsed 5 to 7 times with 5 cc. portions of water.

Clausen titration

The free bisulfite is oxidized to sulfate by adding 0.1 N iodine, 1 cc. of starch solution being used as an indicator, until there is a slight excess of iodine. This excess is removed by the addition of 1 drop of 0.1 N thiosulfate. 0.002 N iodine solution is then added to the point where 1 drop of the iodine produces a clearly discernible change, but not a deep blue, in the colorless solution. The bisulfite combined with aldehyde is then set free by adding a small amount of sodium bicarbonate (enough to discharge the blue color) in either the dry form or as the saturated solution. The sulfite set free is then titrated with the 0.002 N iodine. If there is doubt about the final end-point more bicarbonate is added. If the end-point has been reached the blue color persists for 15 seconds or longer; otherwise it fades quickly.

A blank should be run on the reagents in such a manner as to include

any sulfite-binding material drawn into the apparatus by the air current. It is wise to exclude from the room where determinations are made all substances which may contain acetone or aldehyde vapor. If this can not be done the apparatus must be modified so that in-going air is washed through bisulfite.

Calculation

One cubic centimeter of 1 N iodine is equivalent to 0.5 millimole or 45 mg. of lactic acid. Hence:

$$\text{Millimoles of lactic acid per liter} = \frac{500\ N\ (A - B)}{V}$$

$$\text{Grams of lactic acid per liter} = \frac{45\ N\ (A - B)}{V}$$

where N is the normality of the iodine used for titration, A is the cubic centimeter of iodine solution used to titrate the sulfite bound by aldehyde, B is the cubic centimeters of iodine used in a blank analysis, and V is the cubic centimeters of urine or blood represented in the sample.

When N is 0.002 and the sample represents 2 cc. of *urine*, this calculation becomes:

$$\text{Millimoles of lactic acid per liter urine} = 0.5\ (A - B)$$

$$\text{Grams of lactic acid per liter urine} = 0.045\ (A - B)$$

When the urine must be freed of proteins and the sample represents 10/6 of a cc. the above formulae are changed to 0.6 $(A - B)$ and 0.054 $(A - B)$ respectively.

When the sample represents 5/11 cc. of *blood* the calculation becomes:

$$\text{Millimoles of lactic acid per liter blood} = 2.22\ (A - B)$$

$$\text{Milligrams of lactic acid per 100 cc. blood} = 19.8\ (A - B).$$

Precautions

If the results are to be compared with those normal for resting subjects the subject should avoid muscular exertion for a half-hour before the sample is taken. Exercise may markedly increase the lactic acid content (see volume I).

If the blood is allowed to stand at room temperature the glucose in it is converted to lactic acid by autoglycolysis (7, 15, 16). To prevent this the proteins should be precipitated as early as possible after the blood is withdrawn. If delay can not be avoided the blood should be kept at

refrigerator temperature or treated with 2 mg. of ammonium or potassium fluoride per cubic centimeter.[1]

Many substances, especially hydroxy-acids, yield bisulfite binding substances on oxidation. The reflux condenser reduces this effect by allowing only the more volatile products to be carried over; this is true, however, only if the condenser is kept cool.

COLORIMETRIC METHOD FOR BLOOD. MENDEL AND GOLDSCHEIDER (21)

Principle

The proteins are precipitated by metaphosphoric acid. The filtrate is cleared of carbohydrates by precipitation with copper and lime. The lactic acid is converted by heating with concentrated sulfuric acid into acetaldehyde and carbon monoxide (Deniges (6)). Veratrol (pyrocatechol dimethyl ester, $C_6H_4(OCH_3)_2$) is added, and the color formed by its reaction with acetaldehyde measures the amount of the latter present.

Reagents

Five per cent metaphosphoric acid solution, freshly prepared the same day from glacial phosphoric acid.

Copper sulfate solution. A saturated solution is prepared (about 30 grams of crystalline salt to 100 cc. of water), and is diluted with an equal volume of water.

Calcium hydroxide.

Concentrated sulfuric acid. The sulfuric acid must be tested as follows. To 3 cc. of it 0.1 cc. of the 0.125 per cent veratrol solution is added. If in the course of a few minutes a yellow color develops the acid can not be used. Probably such color is caused by the presence of nitrates or nitrites.

0.125 per cent veratrol solution in absolute alcohol.

Mendel and Goldschneider used Kahlbaum's reagents.

Standard lactate solution. Dissolve in water 171 mg. of crystalline calcium lactate, Ca $(C_3H_5O_3)_2 \cdot 5\ H_2O$, in water in a 100-cc. flask and dilute to the mark. Or, if the anhydrous salt is used, dissolve 121 mg. The solution contains 1 mg. of lactic acid per cubic centimeter.

To make the dilute standard used for comparison in blood analyses, dilute 1 cc. of this solution to 100.

[1] Evans (7) found that potassium fluoride greatly retarded the formation of lactic acid at room temperature. Stadie (personal communication) has found that ammonium fluoride is more efficient, and prevents glycolysis in shed blood even at 38°.

Procedure

Protein precipitation. One cubic centimeter of blood + 6 cc. of water + 1 cc. of metaphosphoric acid are mixed, and shaken vigorously. After the mixture has stood for some minutes it is filtered.

Removal of carbohydrates. Four cubic centimeters of the water-clear, protein-free filtrate are placed in a centrifuge tube. One cubic centimeter of the copper sulfate solution is added, followed by 1 gram of the calcium hydroxide. The mixture is permitted to stand thirty minutes, during which period it is shaken several times. It is then centrifuged.

Decomposition of lactic acid into acetaldehyde. Of the sugar-free supernatant solution 0.5 cc., equivalent to 0.05 cc. of blood, is carefully removed with a pipette and transferred to an absolutely clean and dry test tube. Three cubic centimeters of concentrated sulfuric acid are then added by drops, while the mixture is cooled by shaking in ice water. Then the tube is placed exactly four minutes in boiling water, and at once thereafter cooled in ice water.

At the same time 0.5 cc. of the standard lactic acid solution is treated in the same manner.

Colorimetric determination. After the tubes have cooled two minutes exactly 0.100 cc. of veratrol solution is added and mixed.

After the tubes have stood exactly twenty minutes with veratrol at room temperature the colors are compared.

Calculation

$$\text{Milligrams of lactic acid per 100 cc. blood} = \frac{10\ S}{U}$$

$$\text{Millimoles of lactic acid per liter blood} \quad \frac{1.11\ S}{U}$$

S is the reading of the standard and U that of the unknown.

Precautions

The precautions already mentioned above against formation of lactic acid by exercise of the subject before the blood is drawn and by glycolysis afterwards are to be observed.

It is extremely important that all apparatus used should be absolutely clean. The tubes used for heating the blood filtrate with sulfuric acid

should be cleaned with hot sulfuric acid, rinsed with distilled water, and dried either over a flame or in an oven. They must be completely dry as well as clean.

The sulfuric acid used should be kept in a closed bottle so that its concentration will not be reduced by absorption of moisture from the air.

After centrifugation of the copper-lime mixture it is not permissible to filter through paper, the best of which gives off substances that yield a color. The surface of the solution is often covered with a slight film of copper hydroxide. The pipette for withdrawal of the sample is inserted through this, and the sample is withdrawn without any of the particles. Some of the latter may stick to the outside of the pipette, and are wiped off before the sample is delivered into the final test tube.

Remarks

The reaction appears to be more specific for lactic acid than is the permanganate oxidation. No color is given by acetone, beta-hydroxybutyric acid, acetoacetic acid, urea, uric acid, creatine, creatinine, glycocoll, alanine, or propionic acid. Pyruvic acid and formaldehyde give the reaction, but are not likely to be present. Sugar in even the slightest traces gives the reaction, but is completely removed by the treatment with copper and lime by the Salkowski-Van Slyke method.

The determination can be carried out with 0.5 cc. instead of 1 cc. of blood, the reagents for removal of proteins and carbohydrate being likewise halved.

An artificial standard solution can be made by diluting an alcoholic solution of carbol fuchsin and adding a slight amount of orange G, so that the color matches that obtained with the standard lactate solution.

It is a peculiar fact that the aldehyde does not seem to volatilize from the heated solution. Mendel and Goldschneider obtained a maximum in 4 minutes heating, and no change if the heating was prolonged four minutes more. Hopkins (8) used a similar colorimetric procedure in semi-quantitative tests for lactic acid, in which he heated with sulfuric acid and added thiophene, instead of veratrol, to develop the color. Although acetaldehyde added alone was volatilized when so heated, the substance that gave its color reaction was not.

GASOMETRIC METHOD FOR BLOOD. AVERY AND HASTINGS

This method is described on page 427, in the gasometric chapter.

BIBLIOGRAPHY

1. Baumberger, J. P., and Field, J.: Manometric method for quantitative determination of lactic acid. *Proc. Soc. Exp. Biol. and Med.*, 1927, **25**, 87.
2. Boas, I.: Eine neue Methode der qualitativen und quantitativen Milchsäurebestimmung im Mageninhalt. *Deut med. Woch.*, 1893, **19**, 940.
3. Chelle, L., and Mauriac, P.: Sur la transformation du glucose en acide lactique dans l'autoglycolyse du sang. *Compt. rend. soc. biol.*, 1914, **76**, 852.
4. Clausen, S. W.: A method for the determination of small amounts of lactic acid. *J. Biol. Chem.*, 1922, **52**, 263.
5. Davenport, H. A., and Cotonio, M.: A condenser unit for use in the determination of lactic acod. *J. Biol. Chem.*, 1927, **73**, 359.
6. Denigès, G.: Réactions analytiques de quelques fonctions organiques fondées sur leur transformation en dérivés aldéhydiques et cétoniques. *Ann. chim. phys.*, 1910, (Series viii) **18**, 149.

   Réactions trés sensibles des acides lactique et glycolique. *Bull. Soc. chim.*, 1909, IV **5**, 647.
7. Evans, C. L.: Acid production in shed blood. *J. Physiol.*, 1922, **56**, 146.
8. Fletcher, W. M., and Hopkins, F. G.: Lactic acid in amphibian muscle. *J. Physiol.*, 1907, **35**, 247.
9. Friedemann, T. E.: On the determination of lactic acid in sugar solutions decomposed by alkali. *J. Biol. Chem.*, 1928, **76**, 75.
10. Friedemann, T. E.; Cotonio, M., and Shaffer, P. A.: The determination of lactic acid. *J. Biol. Chem.*, 1927, **73**, 335.
11. Friedemann, T. E., and Kendall, A. I.: The determination of lactic acid. *J. Biol. Chem.*, 1929, **82**, 23.
12. Fürth, O. von, and Charnass, D.: Über die quantitative Bestimmung der Milchsäure durch Ermittlung der daraus abspaltbaren Aldehydmenge. *Biochem. Z.*, 1910, **26**, 199.
13. Harrop, G. A., Jr.: A method for the estimation of lactic acid in blood. *Proc. Soc. Exp. Biol. Med.*, 1919–20, **17**, 162.
14. Jerusalem, E.: Über ein neues Verfahren zur quantitativen Bestimmung der Milchsäure in Organen und tierischen Flüssigkeiten. I. Bestimmung der Milchsäure in wässerigen Lösungen. *Biochem. Z.*, 1908, **12**, 361.

   II. Bestimmung der Milchsäure in tierschen Flüssigkeiten. *Ibid.*, 379.
15. Kondo, K.: Über Milchsäurebildung im Blute. III. *Biochem. Z.*, 1912, **45**, 88.
16. Kraske, B.: Über Milchsäurebildung im Blute. II.: *Biochem. Z.*, 1912, **45**, 81.
17. Long, C. N. H.: Muscular exercise, lactic acid, and the supply and utilization of oxygen. II. The estimation of lactic acid in biological fluids. *Proc. Roy. Soc.*, 1924, B, **96**, 444.
18. Long, C. N. H.: Lactic acid in the blood of a resting man. *J. Physiol.*, 1924, **58**, 455.
19. Maver, Mary C.: The Schneyer method for determination of lactic acid in urine. *J. Biol. Chem.*, 1917, **32**, 71.
20. Meissner, R.: Über die quantitative Bestimmung der Milchsäure in Organextraxten als Kohlenoxyd. *Biochem. Z.*, 1915, **68**, 175.
21. Mendel, B., and Goldscheider, I.: Eine kolorimetrische Mikromethode zur quantitativen Bestimmung der Milchsäure im Blut. *Biochem. Z.*, 1925, **164**, 163.

22. Ripper, M.: Eine allgemein anwendbare, massanalytische Bestimmung der Aldehyde. *Monatshft. Chem.*, 1900, **21**, 1079.
23. Ronzoni, E., and Wallen-Lawrence, Z.: Determination of lactic acid in blood. *J. Biol. Chem.*, 1927, **74**, 363.
24. Ryffel, J. H.: A new method for the estimation of lactic acid in urine. *J. Physiol.* (Soc. Proc.) 1909–10, **39**, p.v.
25. Schneyer, J.: Eine Methode zur quantitativen Milchsäurebestimmung im Harne. *Biochem. Z.*, 1915, **70**, 294.
26. Vas, M., and Lang, A.: Beiträge zum Mendel-Goldscheiderschen Blutmilchsäurebestimmungsverfahren. *Biochem. Z.*, 1927, **172**, 428.
27. West, E. S.: An improved lactic acid apparatus. *J. Biol. Chem.*, 1931, **92**, 483.
28. Wolf, C. G. L.: Determination of lactic acid. *J. Physiol.*, 1914, **48**, 341.

CHAPTER XVII

Acetone, Acetoacetic Acid, and Beta-hydroxybutyric Acid

PRINCIPLES OF METHODS

Under the term "acetone bodies" are included by usage in physiological chemistry acetone, acetoacetic acid, and β-hydroxybutyric acid. Meticulously considered, the term is a misnomer: the things indicated by it are substances rather than bodies; and only one of these is acetone. "Acetone bodies," however, has justified its employment by long survival as a convenient name for the group.

Physiologically these substances are related in that they are formed together in the organism whenever oxidation of fat occurs without the simultaneous oxidation of at least one-half molecule of glucose for each molecule of fatty acid (17) Chemically, acetoacetic acid is an oxidation product of β-hydroxybutyric acid, and acetone is formed by spontaneous decomposition of acetoacetic acid, as indicated in the diagram below.

CH_3		CH_3		CH_3
|		|		|
CH(OH)		CO		CO
CH_2	oxidation by dichromate	CH_2	heat	CH_3
|		|		
COOH		COOH		CO_2
β-hydroxybutyric acid		Acetoacetic acid		Acetone and carbon dioxide

β-hydroxybutyric acid has been extracted from urine by organic solvents and determined by means of the polariscope (3, 11) As a rule, however, it is determined as acetone after conversion by oxidation and heat (16). The latter method only has been applied to blood. Acetoacetic acid is decomposed almost instantly at the boiling temperature into acetone, and all methods for its determination are based on this decomposition. Preformed acetone can be separated from acetoacetic acid by driving off the acetone with a current of cold air (7) into iodine solution, and determined by titration.

Methods used most for the determination of acetone have depended on the following four reactions.

1. *Messinger's iodometric iodine titration* (13). The acetone is distilled into a solution of alkaline iodine which reacts to form iodoform with the utilization of 6 atoms of iodine for each molecule of acetone, as indicated by the following equation:

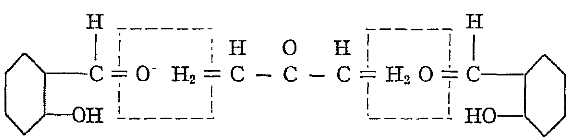
$$CH_3COCH_3 + 6\ I + 4\ KOH = CH_3COOK + CHI_3 + 3\ KI + 3\ H_2O.$$

Because 1 molecule of acetone reacts with 6 molecules of iodine, and iodometric titration can be made in very dilute solutions, this reaction is well suited to micro analyses. One cubic centimeter of 1 N iodine reacts with 9.67 mg. of acetone. Iodine as dilute as 0.001 N can be used, of which 1 cc. titrates approximately 0.01 mg. of acetone (10).

2. *Scott-Wilson's precipitation with mercuric cyanide.* Scott-Wilson (15) worked out the conditions required for quantitative precipitation of acetone with mercuric cyanide and alkali by a reaction which had been discovered by Marsh and Struthers. The precipitate, which is extremely insoluble in alkaline solution, was redissolved in nitric acid, and the mercury was titrated with standard sulfocyanate solution. Marriott (12), and Folin (8) substituted for titration nephelometric estimation of the precipitate. This permits determination of very small amounts, but in the experience of the authors is difficult to perform with any accuracy.

3. *Denigès' precipitation with mercuric sulfate.* Denigès (5) showed that acetone boiled with mercuric sulfate and sulfuric acid forms a crystalline precipitate weighing twenty times as much as the acetone. Acetone can be determined either by weighing the precipitate or by dissolving it and titrating the mercury. Van Slyke (19) and Van Slyke and Fitz (20) have applied the method to analyses of urine and bloods.

4. *Color formation with salicylaldehyde and alkali.* Fabinyi (6) in 1900 showed that warming with alkali causes condensation of 1 molecule of acetone with 2 of salicylaldehyde, with formation of the red dye, dihydroxybenzal-acetone.



Frommer (9) applied the reaction to qualitative detection of acetone in urine, and found that he could detect acetone in a dilution of 0.01 mg. per cubic centimeter. Csonka (4) devised a quantitative colorimetric technique for urine analysis. The procedure has been further developed by Behre

and Benedict (1, 2) who applied it not only to preformed acetone in urine, but also to total acetone bodies in both blood and urine.

The colorimetric procedure suffers at present under one disadvantage. Some preparations of salicylaldehyde, even when redistilled and apparently pure, do not react satisfactorily, and the cause is still unknown.

Choice of methods

The smallest amount of acetone that can be determined with about ±10 per cent accuracy by each of the three methods to be described is indicated by the following.

Messinger-Hubbard titration, 0.01 mg. titrated by 1 cc. of 0.001 N iodine.

Behre-Benedict colorimetric method, 0.025 mg. determined in blood analysis by comparison with most dilute standard.

Denigès-Van Slyke gravimetric method, 0.05 mg. of acetone measured by 1 mg. of precipitate.

With regard to specificity, and consequent simplicity and ease of application, the methods rank in reverse order. The Messinger iodine titration is affected by so many substances that in determination of hydroxybutyric acid or total acetone substances in blood or urine two or three, and in blood four distillations are required to free the acetone from interfering substances before the titration can be applied. The salicylaldehyde color reaction is more specific, and only one distillation is required to separate the acetone from interfering substances. The Denigès-Van Slyke mercuric sulfate precipitation is so specific that it is carried out directly in blood or urine filtrates. Also the Van Slyke hydroxybutyric acid determination is carried out with only one addition of dichromate, while the other methods require repeated additions during a carefully regulated distillation.

For combined simplicity and accuracy the Denigès-Van Slyke precipitation appears to be the method of choice when, as is ordinarily the case in clinical work, one wishes to determine the presence or extent of a definite ketosis. The colorimetric method is an alternative procedure. These procedures suffice to measure any abnormal increases in the ketones in blood or urine. When, however, one desires to measure physiological variations in the slight amounts of ketone bodies that occur in blood or urine under normal metabolic conditions, or to study ketosis with minimum samples of blood, the sensitive iodometric method as applied by Hubbard is the one of choice.

In clinical studies of ketosis the "total acetone bodies" determination is usually the one which yields the desired information.

GRAVIMETRIC AND TITRIMETRIC DETERMINATIONS OF ACETONE BODIES IN URINE BY VAN SLYKE'S METHOD WITH DENIGÈS' REAGENT (19)

Reagents

Twenty per cent copper sulfate. Two hundred grams of $CuSO_4 \cdot 5\ H_2O$ dissolved in water and made up to 1 liter.

Ten per cent mercuric sulfate. Seventy-three grams of pure red mercuric oxide dissolved in 1 liter of H_2SO_4 of 4 N concentration.

Fifty volume per cent sulfuric acid. Five hundred cubic centimeters of sulfuric acid of 1.836 specific gravity, diluted to 1 liter with water. Concentration of H_2SO_4 must be readjusted if necessary to make it 17.0 N by titration.

Ten per cent calcium hydroxide suspension. Mix 100 grams of Merck's fine, light, "reagent" $Ca(OH)_2$ with 1 liter of water.

Five per cent potassium dichromate. Fifty grams of $K_2Cr_2O_7$ dissolved in water and made up to 1 liter.

Combined reagents for total acetone body determination. One liter of the above 50 per cent sulfuric acid, 3.5 liters of the above 10 per cent mercuric sulfate, 10 liters of water.

Preservatives other than toluene or copper sulfate should not be used with the urine.

Removal of glucose and other interfering substances from urine by precipitation with copper and lime

Place 25 cc. of urine in a 250-cc. measuring flask. Add 100 cc. of water, 50 cc. of copper sulfate solution, and mix. Then add 50 cc. of 10 per cent calcium hydroxide, shake, and test with litmus. If not alkaline, add more calcium hydroxide. Dilute to the mark and let stand at least one-half hour for glucose to precipitate. Filter through a dry folded filter. This procedure will remove up to 8 per cent of glucose. Urine containing more should be diluted enough to bring the glucose down to 8 per cent. The copper treatment is depended upon to remove interfering substances other than glucose, and should therefore ***never be omitted, even when glucose is absent.*** The filtrate may be tested for glucose by boiling a little in a test-tube. A precipitate of yellow cuprous oxide will be obtained if the removal has not been complete. A slight precipitate of white calcium salts always forms, but does not interfere with the detection of the yellow cuprous oxide.

Determination of acetone and acetoacetic acid

Acetoacetic acid is decomposed in boiling solution to CO_2 and acetone. This acetone and the preformed acetone are precipitated together by mercuric sulfate.

Place in a 500-cc. Pyrex Erlenmeyer flask 25 cc. of urine filtrate. Add 100 cc. of water, 10 cc. of 50 per cent sulfuric acid, and 35 cc. of the 10 per cent mercuric sulfate. Or in place of adding the water and reagents separately, add 145 cc. of the "combined reagents." Connect the flask with a reflux condenser having a straight condensing tube of 8 or 10 mm. diameter and heat to boiling. Boil gently for thirty minutes, and no longer. Longer boiling than forty-five minutes splits off a little acetone from the hydroxybutyric acid which is always present.

The precipitate may either be filtered at once from the hot solution or after the latter has cooled. The precipitate is collected on a Gooch or porous bottomed glass crucible and washed with about 200 cc. of cold water. Filtration of the coarsely granular precipitate is quick.

For gravimetric determination the crucible and precipitate are dried for an hour at 110°. The crucible is cooled in room air and weighed. Several precipitates may be collected one above the other without cleaning the crucible.

As an alternative to weighing, the precipitate may be dissolved in 1 N hydrochloric acid and titrated as described later. If this is to be done one may, instead of washing the precipitate in a porous crucible, wash it on a small quantitative filter paper without suction.

Simultaneous determination of total acetone bodies (acetone, acetoacetic acid, and hydroxybutyric acid), in one determination

Place in a 500-cc. Pyrex Erlenmeyer flask 25 cc. of urine filtrate. Add water and mercuric sulfate and heat to boiling exactly as in the above described determination of acetone and acetoacetic acid. ***After*** boiling has begun add 5 cc. of the dichromate through the condenser. Continue boiling gently for one and one-half hours. The yellow precipitate which forms consists of the mercury sulfate-chromate compound of the preformed acetone, the acetone which has been formed by heat decomposition of the acetoacetic acid, and the acetone which has been formed by dichromate oxidation of the hydroxybutyric acid.

The precipitate is collected and weighed or titrated as described above for acetone and acetoacetic acid.

The oxidation of β-hydroxybutyric acid to acetone is not complete. Part of the β-hydroxybutyric acid is oxidized to acetic acid instead of to acetone

(19). Under the conditions outlined above, one molecule of β-hydroxybutyric acid yields 0.75 molecule of acetone. If the oxidation occurs in the cold, however, the greater part of the product is acetic acid. For this reason *the dichromate must not be added until the solution has been raised to the boiling point*, as directed.

Normal urines give precipitates of 1 to 20 mg. in this total acetone bodies determination.

β-hydoxybutyric acid

The β-hydroxybutyric acid alone is determined exactly as total acetone bodies except that the preformed acetone and the acetone from the acetoacetic acid are first boiled off. To do this the 25 cc. of urine filtrate plus 100 cc. of water are treated with 2 cc. of the 50 per cent sulfuric acid and boiled in the open flask for ten minutes. The volume of solution left in the flask is measured in a cylinder. The solution is returned to the flask and the cylinder washed with enough water to replace that boiled off and restore the volume of the solution to 127 cc. Then 8 cc. of the 50 per cent sulfuric acid and 35 cc. of mercuric sulfate are added. The flask is connected under the condenser and the determination is continued as described for total acetone bodies.

Blank determination of precipitate from substances in urine other than the acetone bodies

A 25 cc. aliquot of urine filtrate is treated with sulfuric acid and water and boiled ten minutes to drive off acetone as in the above β-hydroxybutyric acid determination. The residue is made up to 175 cc. with the same amounts of mercuric sulfate and sulfuric acid used in the above determinations, but without chromate, and is boiled under the reflux for forty-five minutes. Longer boiling splits off some acetone from β-hydroxybutyric acid, and must therefore be avoided. The weight of precipitate obtained may be subtracted from that obtained in the above determination. The blank is so small (from 1 to 9 mg.), that in our experience it is relatively significant only when compared with the small amounts of acetone bodies found in normal or nearly normal urines. In routine analyses of diabetic urines we do not determine it.

Test of reagents

When the complete total acetone bodies determination, including the preliminary copper sulfate treatment, is performed on a sample of distilled water instead of urine no precipitate whatever should be obtained. This test must not be omitted.

Calculation

One milligram of β-hydroxybutyric acid yields 8.45 mg. of precipitate.

One milligram of acetone yields 20.0 mg. of precipitate.

One cubic centimeter of 0.2 M KI solution is equivalent to 1.3 mg. of precipitate in titration of the latter.

From these values the factors of table 55 have been calculated.

TABLE 55

FACTORS FOR ACETONE BODIES IN URINE BY VAN SLYKE'S GRAVIMETRIC OR TITRATION METHOD

For use when 25 cc. of urine filtrate, equivalent to 2.5 cc. of urine, are used for a determination

DETERMINATION PERFORMED	FACTORS BY WHICH GM. OF PRECIPITATE ARE MULTIPLIED TO CALCULATE ACETONE BODIES IN TERMS OF		FACTORS BY WHICH CUBIC CENTIMETERS OF 0.2 M KI ARE MULTIPLIED TO CALCULATE ACETONE BODIES IN TERMS OF	
	Grams of acetone per liter urine	Millimoles per liter urine	Grams of acetone per liter urine	Millimoles per liter urine
Acetone plus acetoacetic acid	20.0	345	0.260	4.48
β-hydroxybutyric acid.......	26.4	455	0.344	5.93
Total acetone bodies........	24.8	428	0.322	5.55

The "total acetone bodies" factor is calculated on the assumption that the molecular proportion of these bodies in the form of β-hydroxybutyric acid is 75 per cent of the total, which proportion is usually approximated in acetonuria. Because β-hydroxybutyric acid yields only 0.75 molecule of acetone, the factors are strictly accurate only when this proportion is present, but the error introduced by the use of the approximate factors is for ordinary purposes not serious. The actual errors in percentages of the amounts determined are as follows: molecular proportion of acetone bodies as β-acid 0.50, error −6.5 per cent; β-acid 0.60, error −3.8 per cent; β-acid 0.80, error + 1.3 per cent.

In order to calculate the acetone bodies as *β-hydroxybutyric acid* rather than acetone, the acetone factors in table 55 are multiplied by the ratio of the molecular weights

$$\frac{\beta\text{-acid}}{\text{acetone}} = \frac{104}{58} = 1.793.$$

Titration of mercury precipitates (19)

In all the above determinations titration of the mercury in the precipitate may be substituted for weighing the latter. The precipitate is redissolved in hydrochloric acid, and the mercury is titrated by Personne's (14) method. For this titration two successive reactions of $HgCl_2$ with KI are utilized.

$$1.\quad HgCl_2 + 2\ KI = \underset{\text{red precipitate}}{HgI_2} + 2\ KCl$$

$$2.\quad HgI_2 + 2\ KI = \underset{\text{colorless solution.}}{K_2HgI_4}$$

When KI is run into mercuric chloride solution one first observes formation of the red precipitate. Then when with further KI addition, 4 molecules of KI per molecule of $HgCl_2$ have been added, the precipitate redissolves and a clear solution is obtained again. At the end-point adding a drop of mercuric chloride solution will cause a red precipitate and a drop of iodide will redissolve it. The sharper end-point is obtained by adding mercuric salt to iodide, so that appearance of red precipitate marks the point. Hence in the present titration standard KI is added until the HgI_2 first formed redissolves: then the excess KI is titrated back with standard $HgCl_2$ solution until a permanent trace of the precipitate reappears.

The total reaction utilized is therefore:

$$HgCl_2 + 4\ KI = K_2HgI_4 + 2\ KCl,$$

One molecule of $HgCl_2$ reacts with 4 of KI, so that 0.05 *M* $HgCl_2$ solution should titrate an equal volume of 0.20 *M* KI, according to the above equation. Actually about 4.2 molecules of KI are required to titrate one of $HgCl_2$.

Reagents

0.05 M mercuric chloride prepared by dissolving 13.576 grams of mercuric chloride in water and diluting to one liter. The solution is standardized as follows: 25 cc. of 0.05 M mercuric chloride are diluted to about 100 cc., and hydrogen sulfide is run in until the black precipitate flocculates, leaving a a clear solution. The mercuric sulfide is collected in a Gooch crucible, dried at 110°C., and weighed. If the solution is accurate the precipitate should weigh 0.2908 grams.

0.2 M KI solution. Theoretically this solution should contain 33.2 grams of KI per liter. Actually about 5 per cent more than the theoretical 4 molecules of KI is required to titrate the mercury. The iodide solution is therefore made as follows. Thirty-six grams of KI are dissolved in water and made up to 1 liter, and the solution is titrated against the standard 0.05 M $HgCl_2$. 20 cc. of the iodide are measured into a flask and the mercuric chloride is run in until the end point is reached. Somewhat more than 20 cc. of the 0.05 M $HgCl_2$ will be required. From the titration one calculates how much to dilute the iodide solution in order to make it exactly balance

the bichloride solution. Expressing the cubic centimeters of 0.05 M $HgCl_2$ used in titrating 20 cc. of iodide as A, and the volume of iodide solution remaining in the stock flask as V, enough water is added to the stock solution to increase its volume from

$$V \text{ cubic centimeters to } \frac{A}{20} \times V \text{ cubic centimeters.}$$

1 N hydrochloric acid.

3 M sodium acetate. 408 grams of crystalline $NaC_2H_3O_2 \cdot 3H_2O$ or 246 grams of anhydrous $NaC_2H_3O_2$ are dissolved in water and made up to a liter.

Titration procedure

If a Gooch crucible has been used to collect the precipitate it is inverted over a small beaker and the asbestos mat and precipitate are blown into the beaker. Any particles adherent to the crucible walls are washed into the beaker with as little water as possible. Then 15 cc. of 1 N hydrochloric acid are added and the mixture is warmed until the precipitate dissolves. If a porous bottomed alundum or glass crucible has been used it is set into a small beaker with 15 cc. of 1 N hydrochloric acid and heated until the precipitate dissolves. The crucible is then washed with suction by 3 portions of about 5 cc. of water each, the successive washings being added to the beaker. In place of using either a Gooch or porous bottomed crucible one may, when titration is to be employed, wash the precipitate without suction on a small quantitative filter paper, which is transferred with precipitate to the beaker and broken up with a rod in the 15 cc. of 1 N acid, which is then warmed to dissolve the precipitate.

In order to obtain a good end-point in the subsequent titration, it is necessary to reduce the acidity of the solution. For this purpose, 6 to 7 cc. of 3 M sodium acetate solution are added to the solution after it has been cooled. 0.2 M potassium iodide is then run in rapidly from a burette with constant stirring. If more than a small amount of mercury is present, a red precipitate of mercuric iodide at once forms, but redissolves as soon as 2 or 3 cc. of potassium iodide in excess of the amount required to form the soluble potassium mercuric iodide have been added. Addition of potassium iodide is then stopped. If only a few milligrams of mercury are present, the excess of potassium iodide may be added before the mercuric iodide has had time to precipitate, so that the titrated solution remains clear. In this case 5 cc. of potassium iodide are added, as it has been found that the final titration is not satisfactory if

less is present. The excess of potassium iodide is titrated back by adding 0.05 M mercuric chloride from another burette until a permanent red HgI_2 precipitate forms.

For small precipitates the volume of 1 N hydrochloride acid and acetate solutions used may be reduced to one-half or one-third that above directed, with corresponding sharpening of the end-point.

Calculation

$$(A - B) \times factor = \text{acetone bodies.}$$

A represents cubic centimeters of 0.2 M potassium iodide and B the cubic centimeters of 0.05 M mercuric chloride used in the titration. The values of the *factor* for the different analyses are given in tables 55 and 56. These factors are calculated by utilizing the fact that the mercuric-sulfate-acetone precipitate is 76.9 per cent mercury (19) whether chromate is present or absent during the precipitation. Each cubic centimeter of 0.2 M potassium iodide solution is equivalent to 10.0 mg. of Hg, and hence to $\frac{10.0}{0.769} = 13.0$ mg. of mercuric-sulfate-acetone precipitate, and to $\frac{13}{20} = 0.65$ mg. of acetone.

COLORIMETRIC DETERMINATION OF ACETONE BODIES IN URINE. BEHRE AND BENEDICT (2)

Reagents

For the removal of sugar and other interfering substances by the Van Slyke method (with modified concentrations). Copper sulfate, 40 per cent solution, or powdered in a mortar. Calcium hydroxide, 20 per cent suspension, or dry.

For the oxidation of β-hydroxybutyric acid by the Shaffer-Hubbard method. Fifty per cent concentrated sulfuric acid made by adding 1 volume of concentrated acid to 1 volume of water. 0.2 per cent potassium dichromate solution.

For the colorimetric determination of acetone. Sodium hydroxide, 32 per cent solution. Salicylicaldehyde.

Behre and Benedict found great differences in the delicacy of the color reaction given by different samples of salicylaldehyde. A sample of Kahlbaum's technical salicylaldehyde proved very satisfactory, as did also Eimer and Amend's high grade product labelled "Acid salicylous, synthetic (salicylicaldehyde)." Samples from the Eastman laboratory, though less deeply colored than the Eimer and Amend product, did not yield nearly so deep a color with a given amount of acetone as did the other samples used.

Standard acetone solutions

Stock solution. This contains 0.1 mg. of acetone per cubic centimeter. It is most easily prepared from a solution containing 1 cc. of acetone in 1 liter of water, whose actual acetone content by weight has been determined by titration with 0.1 N iodine as described on page 638. The stock solution should be prepared from this to contain 0.1 mg. of acetone per cubic centimeter. This solution can be kept for about a month without deterioration.

Standard solution. By 1:10 dilution of the stock solution a standard solution, containing 0.01 mg. per cubic centimeter, is prepared for use in the actual determination. It is best to make up this dilute solution every second day, and to keep it will corked when not in use.[1]

Acetone and Acetoacetic Acid

Such a volume of urine as will contain about 0.1 mg. of acetone (usually from 2 to 50 cc. are required), is transferred to a 100 or 150-cc. distilling flask, the volume made up to about 75 cc. with distilled water, and 3 or 4 drops of sulfuric acid, diluted 1:1, are added. The flask is tightly fitted with a cork stopper and connected with a water-cooled condenser. The condenser is provided with a bent glass tube which has been drawn out long enough to a sufficiently small diameter to reach to the bottom of a 25 or 50 cc. volumetric flask, and which dips below the surface of a minimum amount of water in the flask. None of the connections should be of rubber. Rubber stoppers covered with tin-foil, or cork stoppers, often renewed, can be used. The preformed acetone and acetone from diacetic acid are then distilled into the 25 or 50-cc. flask. Except when very large amounts of acetone are present a distillation to 25 cc. gives good results. When the distillate has almost reached the volume desired, the bent tube is disconnected and washed out with a few drops of water, and the distillate made up to volume and mixed. Five cubic centimeters of the distillate are transferred to a test tube and exactly 5 cc. of a 32 per cent solution of sodium hydroxide and 10 drops of salicylic aldehyde are added. Standards are prepared in test tubes at the same time from the dilute (0.01 mg. per cubic centimeter) acetone solution. By using from 0.5 to 5 cc. of this solution a range of standards containing from 0.005 to 0.05 mg. can be made. Unless the approximate acetone content of the unknown solution is known, standards containing 0.005, 0.01, 0.02, 0.03, and 0.05 mg. had best be

[1] Behre (1) has prepared for approximate determinations permanent artificial standards by diluting a mixture of 1 part of potassium dichromate and 10 parts of cobalt chloride.

made. In each case the volume of the standard solution must be made to 5 cc. Exactly 5 cc. of 32 per cent sodium hydroxide and 10 drops of salicylic aldehyde are also added to each of the standard tubes. The contents of the tubes are mixed by side to side shaking and the tubes then immersed in a boiling water bath for from three to five minutes. If the salicylic aldehyde does not dissolve easily the tubes must be shaken until solution is effected. After the heating the tubes are removed and allowed to cool, the solutions filtered, and colorimetric comparison is made. The standard used should be of such a concentration that the unknown solution gives a reading between 11 and 19 mm. with the standard set at 15 mm.

Calculation

$$\frac{0.2\ S \times A \times D}{U \times V} = \text{grams of acetone in 1 liter urine.}$$

$$\frac{3.45\ S \times A \times D}{U \times V} = \text{millimoles of acetone per liter urine.}$$

S = reading of standard; U = reading of unknown; A = milligrams of acetone in standard; V = cubic centimeters of urine distilled; D = cubic centimeters of total volume of distillate (of which 5 cc. are used for colorimetric measurement).

β-hydroxybutyric acid

If β-hydroxybutyric acid is to be determined in addition to acetone, sugar and other interfering substances must be removed, even from normal urine, before any distillation is made. For this the urine is treated with copper sulfate and calcium hydroxide according to the Van Slyke procedure (19). In order to keep down the volume of solution to be distilled, however, the urine is diluted 1:5 instead of 1:10, using 1 volume of urine, 1 volume of 40 per cent copper sulfate solution, and enough of a 20 per cent suspension of calcium hydroxide to make the reaction alkaline to litmus (probably 1 volume). The whole mixture is then made up to five volumes. If the urine is very low in acetone bodies the copper sulfate can be powdered in a mortar and both this and the calcium hydroxide added in powdered form. The copper sulfate should be dissolved before the calcium hydroxide is added. The mixture must be shaken very thoroughly and allowed to stand for one-half or three-quarters of an hour, with occasional shaking. It is then filtered and a

volume of the filtrate equivalent to from 2 to 50 cc. of urine (depending on the acetone content) placed in a 300-cc. distilling flask and made acid with 3 or 4 drops of 50 per cent sulfuric acid. The volume is made up to about 75 cc., the flask is fitted with a dropping funnel and connected with a water-cooled condenser, and the distillation and determination of acetone and diacetic acid are carried out as described above.

After distillation of the preformed acetone, a 100-cc. receiving flask is substituted for the 25-cc. flask, the residue in the distilling flask is brought to a boil, and 30 cc. of half concentrated sulfuric acid and 20 cc. of 0.2 per cent potassium dichromate are added gradually through the dropping funnel while a slow distillation goes on. Fifty cubic centimeters more of the dichromate are added after ten minutes and 50 cc. more after another interval of ten minutes. The process differs from the Hubbard method only in that the distillation is made very slowly and the volume of distillate kept down to 100 cc. The distillation should occupy at least thirty minutes. When the distillation to 100 cc. is almost complete the receiving apparatus is again disconnected, the bent tube washed down with a little water, and the distillate made up to 100 cc. and mixed.

Calculation

$$\frac{0.2\,S \times A \times D}{U \times V \times 0.85} = \frac{0.235\,S \times A \times D}{U \times V} = \left\{\begin{array}{l}\text{hydroxybutyric acid expressed as grams}\\ \text{of acetone per liter urine.}\end{array}\right.$$

$$\frac{0.425\,S \times A \times D}{U \times V} = \text{grams of hydroxybutyric acid per liter urine.}$$

$$\frac{4.05\,S \times A \times D}{U \times V} = \text{millimoles of hydroxybutyric acid per liter urine.}$$

The letters have the same significance as in the acetone + acetoacetic acid calculation above. The factor 0.85 is introduced because each molecule of hydroxybutyric acid yields only 0.85 molecule of acetone.

Total acetone bodies

The total acetone bodies are determined exactly like the hydroxybutyric acid, except that the preliminary removal of the preformed acetone and acetoacetic acid by distillation before the dichromate is added is left out. Consequently the acetone from these sources is obtained in the same distillation with that from the oxidized hydroxybutyric acid.

Calculation

$$\frac{0.22\ S \times A \times D}{U \times V} = \text{total acetone bodies in grams of acetone per liter of urine.}$$

$$\frac{3.8\ S \times A \times D}{U \times V} = \text{millimoles of total acetone bodies per liter of urine.}$$

The factor 0.91 is introduced on the assumption that 75 per cent of the total acetone bodies are in the form of hydroxybutyric acid, yielding 0.85 mole of acetone, and 25 per cent in the form of acetone and acetoacetic acid, yielding acetone quantitatively (see note below Table 55).

IODOMETRIC DETERMINATION OF ACETONE BODIES IN URINE BY HUBBARD'S[2] (10) MODIFICATION OF SHAFFER'S (17) METHOD

The method depends on the removal of sugar and other interfering substances by alkaline lead acetate and copper sulfate, the transformation of diacetic acid and β-hydroxybutyric acid into acetone by hot sulfuric acid and acid dichromate, and distillation and iodometric titration of the acetone. Each molecule of hydroxybutyric acid yields about 0.85 molecule of acetone by the dichromate oxidation.

Reagents

Goulard's extract. Rub 110 grams of finely powdered lead oxide into a smooth paste with about 100 cc. of water. Dissolve 180 grams of lead acetate in about 700 cc. of boiling water. Add the lead acetate solution slowly to the lead oxide. Boil the mixture in a porcelain dish of about 1 liter capacity for half an hour, stirring occasionally, and adding water as required to maintain the original volume. Cool and filter the solution, protecting it as much as possible from the air. Make the solution up to a weight of 1000 grams with water which has been previously boiled and cooled (U.S.P., 1916, *9*, 249).

A 20 per cent solution of copper sulfate ($CuSO_4 \cdot 5H_2O$).

Sodium hydroxide solution, 2 N.

Fifty per cent sulfuric acid (1 volume concentrated sulfuric acid plus 1 volume water).

A 10 per cent sodium hydroxide solution.

Potassium permanganate crystals.

Sodium peroxide.

[2] Hubbard's analytical methods here given, both for blood and urine, differ in certain details from the procedures originally described. For these changes the authors are indebted to Dr. Hubbard to whom the manuscript was submitted for correction.

Iodine in potassium iodide. A stock 0.1 N solution of iodine containing 25 grams of potassium iodide per liter. (For preparation and standardization see p. 34). This solution is diluted with 2.5 per cent potassium iodide solution, to 0.01 or 0.02 N for use with urines containing but slight amounts of acetone.

Standard thiosulfate solution. A stock 0.1 N solution of sodium thiosulfate (for preparation and standardization see p. 32). One cubic centimeter of 0.1 N iodine titrates 0.9665 mg. of acetone. Hubbard recommends the use of 0.1035 N iodine and thiosulfate, 1 cc. of which titrates exactly 1 mg. of acetone. If, however, thiosulfate and iodine are being used for other purposes it may be more convenient to use solutions of the usual normality.

Starch indicator solution (for preparation see p. 34).

Concentrated sodium hydroxide, 2 parts by weight of NaOH in 3 of water.

0.1 to 0.2 per cent potassium dichromate solution.

Removal of sugar and other easily oxidized compounds

The urine to be examined must not contain more than 5 per cent of sugar. If the concentration of sugar exceeds 5 per cent, therefore, the urine must be diluted in advance of the analysis. Ten cubic centimeters of urine (or diluted urine) are diluted to 100 to 150 cc. in a glass-stoppered 250-cc. graduated cylinder. To this are added 10 cc. of Goulard's extract, 10 cc. of copper sulfate and 10 cc. of 2 N sodium hydroxide. Dilute the mixture to 250 cc. and after it has stood for about thirty minutes filter it.

Acetone and acetoacetic acid

To 150 cc. of the filtrate, in a 300-cc. Kjeldahl flask, add 10 cc. of 50 per cent sulfuric acid. Insert in the flask a two-holed rubber stopper fitted with a dropping funnel in one hole and in the other a bent tube connecting with a condenser. Distill the fluid for ten minutes at such a rate that 50 cc. of distillate comes over in that time. The distillate should be collected in a 500-cc. flask containing a little water, in which the end of the delivery tube must be immersed. Make the distillate up to about 150 cc. Add to the distillate 5 cc. of the 50 per cent sulfuric acid and 0.2 gram of potassium permanganate and redistill into a second 500-cc. flask containing a little water for ten minutes or more, until a final volume of about 100 cc. of redistillate is obtained. Care must be taken that none of the permanganate solution boils over. To the second distillate add about 0.5 gram of sodium peroxide and distill for ten

minutes into an Erlenmeyer flask containing a little water, until 50 to 100 cc. of fluid is collected. For this distillation ***cork stoppers*** should be used and care must be taken at the start or the solution will foam over.

For precise results one should clean the flask from which the final distillation is made by boiling it out with dilute sodium peroxide just before the flask is used. It is also necessary that the laboratory be free from fumes of substances, such as ammonia and formaldehyde, which reduce iodine solution.

When the urine gives a distinctly positive ferric chloride or nitroprusside test, the purification from permanganate is unnecessary. In this case a single distillation from alkali by the method of Shaffer is simpler and reduces the chances of loss of acetone by vaporization. To the first distillate (from acidified filtrate) add about 5 cc. of 10 per cent sodium hydroxide and distill the solution into an Erlenmeyer flask containing a little water. The distillate from the alkaline solution is used, without further treatment, for the determination of acetone.

Into the final distillate are accurately measured 10 to 25 cc. of the iodine-potassium iodide solution, 0.1, 0.02, or 0.01 N, depending on the concentration of acetone in the urine, as indicated by preliminary qualitative tests. Then 2 cc. of concentrated sodium hydroxide are added, and the solution is mixed thoroughly and allowed to stand for ten minutes or more. It is now acidified with sulfuric acid and, after about five minutes, is titrated with thiosulfate of a concentration equivalent to that of the iodine used. A small amount of starch solution is added before the end-point is reached, to serve as indicator.

Calculation

$$\frac{9.67\ \text{N}\ (A - B - C)}{V} = \left\{\begin{array}{l}\text{acetone + acetoacetic acid calculated}\\ \quad \text{as grams of acetone per liter urine.}\end{array}\right.$$

$$\frac{166.7\ \text{N}\ (A - B - C)}{V} = \left\{\begin{array}{l}\text{millimoles acetone + acetoacetic}\\ \quad \text{acid per liter of urine.}\end{array}\right.$$

N is the normality (0.1, 0.02, or 0.01) of the iodine and thiosulfate used, A = the cubic centimeters of standard iodine used, B = cubic centimeters of thiosulfate used, C is the $A - B$ value obtained in a blank analysis, and V = the cubic centimeters of undiluted urine used for analysis. $V = 6$, if the above directions are followed.

β-hydroxybutyric acid

After acetone and acetoacetic acid have been removed by distillation as above described, replace the receiving flask by a second Kjeldahl flask containing a little water in which the end of the delivery tube is immersed. Then reheat the urine solution to boiling and add through the dropping funnel to the boiling solution 20 cc. of 50 per cent sulfuric acid and 30 cc. of 0.1 to 0.2 per cent potassium dichromate. Boil at such a rate that about 50 cc. distill in ten minutes. After ten minutes add 50 cc. of the same dichromate. Continue boiling and, after ten minutes, add another 50 cc. of dichromate. Continue boiling ten minutes longer.

The distillate is then redistilled successively from acid permanganate and from sodium peroxide in the manner described for the determination of acetone plus acetoacetic acid. If much acetone is present the redistillation from acid permanganate may be omitted and that from sodium peroxide alone may be used.

The determination in the last distillate obtained is carried out as described for acetone plus acetoacetic acid. The value obtained gives the acetone from the oxidation of β-hydroxybutyric acid.

Calculation

$$\frac{11.4 \times (A - B - C)}{V} = \left\{\text{hydroxybutyric acid calculated as grams acetone per liter urine.}\right.$$

$$\frac{20.4 \times (A - B - C)}{V} = \left\{\text{grams of hydroxybutyric acid calculated as such per liter urine.}\right.$$

$$\frac{197 \times (A - B - C)}{V} = \text{millimoles of hydroxybutyric acid per liter urine.}$$

The letters in these formulas have the same significance as in the foregoing formula for calculation of acetone and acetoacetic acid. Here the factor 11.4 replaces 9.67 in order to allow for the fact that each molecule of hydroxybutyric acid yields only 0.85 molecule of acetone.

Total acetone bodies

If it is desired to determine acetone from all the acetone bodies simultaneously, acetone from preformed acetone and diacetic acid is not distilled off before the addition of dichromate is begun. The acetone from all three acetone substances is collected in one distillate, and is redistilled from sodium peroxide, or successively from permanganate and peroxide, as in the foregoing description.

Calculation

$$\frac{11 \times (A - B - C)}{V} = \begin{cases}\text{grams of total acetone bodies calculated as acetone per liter} \\ \text{of urine.}\end{cases}$$

The factor 11 is correct when 75 per cent of the acetone bodies are hydroxybutyric acid, which is about the average proportion in marked ketosis (see discussion of table 55).

$$\frac{190 \times (A - B - C)}{V} = \text{millimoles of total acetone bodies per liter urine.}$$

GRAVIMETRIC OR TRITRIMETRIC DETERMINATION OF THE ACETONE BODIES IN BLOOD WITH DENIGÈS' REAGENT. VAN SLYKE AND FITZ (20)

Reagents

The *10 per cent mercuric sulfate, 50 per cent sulfuric acid,* and *5 per cent potassium dichromate* described on page 626 for determination of ketones in urine.

Removal of proteins from whole blood

Ten cubic centimeters of whole blood are diluted with about 100 cc. of water in a 250-cc. volumetric flask, and 20 cc. of the 10 per cent mercuric sulfate are added. The solution is shaken for a moment, until the protein coagulates, and is then diluted with water up to the 250-cc. mark. If the blood is diluted with much more than 10 volumes of water before the mercury is added, coagulation of the proteins is considerably slower. After fifteen minutes or longer it is filtered through a dry folded filter. If the first drops are cloudy they are passed through the filter a second time. The filtrate has a slight pink tinge, but the substance responsible for this is not precipitated when boiled with mercuric sulfate, and does not interfere with any of the acetone body determinations.

Removal of proteins from plasma or serum

Eight cubic centimeters of oxalated plasma or of serum are diluted in a 200-cc. flask with 50 cc. of water and 15 cc. of the 10 per cent mercuric sulfate are added. The flask is shaken for a moment, until the fine precipitate which first forms has flocculated, and is then filled to the mark with water. After standing fifteen minutes or longer the solution is filtered.

Separate determinations of acetone plus acetoacetic acid and of β-hydroxybutyric acid in a single portion of blood filtrate

Place 125 cc. of protein-free filtrate, representing 5 cc. of either whole blood or plasma, in a 500-cc. flask of Pyrex glass, and to it add 10 cc. of 50 per cent sulfuric acid and 35 cc. of 10 per cent mercuric sulfate. The mixture is heated to boiling under a reflux condenser having a straight condensing tube of 8 or 10 mm. inner diameter, and is boiled gently for thirty minutes. The hot solution is decanted off the precipitate through a Gooch or porous glass crucible with slight suction, and the filtrate is caught in a clean dry flask.

After as much of the solution as possible has been filtered through, and before any wash water has been used, 160 cc. of the filtrate, equivalent to 4.7 cc. of blood, are transferred to another 500-cc. Erlenmeyer flask. This filtrate is heated to boiling under a reflux condenser, and 5 cc. of 5 per cent potassium dichromate solution are added through the condenser. The determination of *β-hydroxybutyric* acid is carried out from this point as described for total acetone bodies in urine.

In the mean time the precipitate obtained from *acetone and acetoacetic acid* during the first thirty minutes boiling is collected in the crucible, washed with 200 cc. of cold water, and determined either by weighing or titration.

Total acetone bodies

Place 125 cc. of filtrate, equivalent to 5 cc. of blood or plasma, in a 500-cc. Pyrex Erlenmeyer flask, add 10 cc. of 50 per cent sulfuric acid and 35 cc. of 10 per cent mercuric sulfate, and heat to boil under a reflux condenser as described for preceding analysis. When boiling has begun add 5 cc. of dichromate solution through the condenser. The remainder of the determination is as described on page 627 for total acetone bodies in urine.

In normal blood the total acetone bodies give only 1 or 2 mg. of precipitate.

The mercury precipitates from all the blood acetone bodies must be filtered while the solutions are still hot, although the washing is done, as in the case of the urine precipitates, with cold water. If the solution and precipitate in a blood analysis are allowed to stand and cool before filtration is begun, a slight amount of flocculent precipitate may form from substances other than acetone, and cause a plus error in the results.

Ether-containing blood. Short (18) has found that the amount of ether

in the blood of an anesthetized person may be sufficient to give a precipitate when boiled with mercuric sulfate and dichromate. The filtrate from such blood should be freed from ether by boiling for a few minutes, or by drawing a rapid current of air through it, before it is used for acetone body determination.

Titration of the precipitate as described on page 629, may be used in place of weighing if the concentration of acetone bodies is as much as 1 millimolar, sufficient to take over 0.3 cc. of the standard KI solution. This is the case whenever ketosis is of clinical significance.

Calculation

The calculation factors given in table 56 are used. The principles of the calculation are the same as described for the calculation of urine results on page 629.

TABLE 56

FACTORS FOR ACETONE BODIES IN BLOOD BY THE VAN SLYKE-FITZ GRAVIMETRIC OR TITRATION METHOD*

For use when filtrate equivalent to 5 cc. of blood is used for a determination

DETERMINATION PERFORMED	FACTORS BY WHICH MILLIGRAMS OF PRECIPITATE ARE MULTIPLIED TO CALCULATE ACETONE BODIES IN TERMS OF		FACTORS BY WHICH CUBIC CENTIMETERS OF 0.2 M KI ARE MULTIPLIED TO CALCULATE ACETONE BODIES IN TERMS OF	
	Milligrams of acetone per 100 cc. blood	Millimoles acetone per liter blood	Milligrams of acetone per 100 cc. blood	Millimoles acetone per liter blood
Acetone plus acetoacetic acid....	1.00	0.172	13.0	2.24
β-hydroxybutyric acid..........	1.32 (1-40)	0.228 (0.141)	17.2	2.96
Total acetone bodies...........	1.24	0.214	16.1	2.78

* The factors in parentheses are used when β-hydroxybutyric acid is determined in the filtrate from the precipitated acetone and acetoacetic acid as described. In this case the amount of filtrate taken for the β-acid determination is equivalent to only $\frac{160}{170}$ of 5 cc. of blood, and the factor must be correspondingly increased.

COLORIMETRIC DETERMINATION OF ACETONE BODIES IN BLOOD. BEHRE AND BENEDICT (2)

Reagents

Same described for Behre-Benedict urine analysis.

Acetone and acetoacetic acid

The blood proteins are precipitated by the regular Folin-Wu method (see p. 65) making a dilution of the blood of 1:10. From 10 to 100 cc.

of the filtrate, depending on the acetone content, are transferred to a 300-cc. distilling flask, 3 or 4 drops of 50 per cent sulfuric acid are added, the volume is made up to 50 to 75 cc., and distillation carried out as described for the determination in urine. The distillation is made into a 20-cc. receiving flask or graduated test tube unless the acetone content of the amount of blood used is known to be high (above 0.05 mg.), in which case the distillation is made into a 25-cc. flask or graduated tube. If the amount of acetone in the filtrate used is known to be 0.1 mg., or more, the distillate may be made to 50 cc. In any case the distillation is stopped just before the desired volume has been reached, the distillate is made up to volume, and 5 cc. of the distillate are heated with alkali and salicylic aldehyde, cooled, and read in a colorimeter as described for the urinary determination.

β-hydroxybutyric acid

1. If the actual volume of filtrate used is known to contain enough β-hydroxybutyric acid to yield 0.1 mg. of acetone or more, the distillation is carried out as described for the determination in urine, the volume of the distillate being kept within 100 cc., and finally made up to 100 cc. The colorimetric determination is made upon 5 cc. of the distillate, as described above for urine.

2. If the volume of filtrate used is expected to contain less than 0.1 mg., the distillation is carried out without regard to the volume of distillate collected, and after the thirty-minute period, the distillate is redistilled into a 20, 25, or 30-cc. volumetric flask (or graduated test tube), according to the amount of acetone expected. In any case the distillate is made up to volume and the acetone content of 5 cc. of the distillate determined as described above.

The formulae given in the section on urinary determination may be used for all the calculations.

IODOMETRIC DETERMINATION OF ACETONE BODIES IN BLOOD. HUBBARD (10)

The general principles of the method and special reagents required are described in the procedure for analysis of urine.

Reagents

The only reagent required other than those used in the analysis of urine (see above) is *colloidal iron solution,* (Merck's dialyzed iron, with 5 per cent Fe_2O_3) for precipitation of proteins.

Precipitation of proteins and other interfering compounds

From 1 to 5 cc. of blood are measured into a 100-cc. glass-stoppered graduated cylinder and enough water is added to bring the volume to between 40 and 50 cc. If more than 5 cc. of blood is available (up to 15 cc.) carry out separate precipitations and combine the filtrates. After the addition of 10 cc. of colloidal iron the solution is well shaken. Ten cubic centimeters of Goulard's extract are next added and the mixture is again shaken. Finally enough 2 N sodium hydroxide is introduced to precipitate the lead, the solution is again mixed and then is allowed to stand for about one hour. After this the mixture is centrifuged in tubes covered with rubber caps to prevent loss of acetone. The supernatant liquid is then filtered. The amount of sodium hydroxide necessary must be determined by preliminary experiment for each lot of Goulard's extract, because the lead content of the reagent is variable, but is usually 5.5 to 6 cc. The amount to use is the smallest quantity which will precipitate all the lead from solution. The filtrate from the lead precipitation should be alkaline to litmus, but only faintly alkaline to phenolphthalein. Only about 2 cc. of 0.01 N acid should be required to render 50 cc. of the filtrate neutral to phenolphthalein.

Acetone and acetoacetic acid

The determination is made by using 150 cc. of the filtrate, or filtrate + water to make a volume of 150 cc. Distillation of the acetone is carried out as described in the analysis of urine above with one exception. Greater accuracy can be obtained if the acetone is distilled from alkali by the method of Shaffer before it is subjected to the action of permanganate and sodium peroxide. Therefore for the determination of acetone and diacetic acid four distillations are necessary: 1, from acidified solution; 2, from alkaline solution; 3, from acid + permanganate; 4, from sodium peroxide.

The determination is carried out as described under the method for urine, and the precautions discussed there must be observed; i.e., a control titration of the alkaline iodine solution must be carried through and the final value corrected for the blank given by the reagents. It is usually advisable to use 10 cc. of 0.002 N iodine solution and titrate the excess with 0.01 N thiosulfate.

Calculation

Calculations are the same given above for the Shaffer-Hubbard method with urine.

β-hydroxybutyric acid and total acetone bodies

The determinations of β-hydroxybutyric acid and of total ketones are carried out on the blood filtrate exactly as described for urine, except that a distillation from alkali is added between the initial distillation from acid dichromate and the redistillation from acid permanganate, as described above for the determination of acetoacetic acid in blood. The final titration and calculation are performed as described above.

BIBLIOGRAPHY

1. Behre, J. A.: A rapid quantitative method for the determination of acetone and diacetic acid in urine. *J. Lab. Clin. Med.*, 1928, **13**, 1155.
2. Behre, J. A., and Benedict, S. R.: A colorimetric method for the determination of acetone bodies in blood and urine. *J. Biol. Chem.*, 1926, **70**, 487.
3. Black, O. F.: The detection and quantitative determination of β-oxybutyric acid in the urine. *J. Biol. Chem.*, 1908–09, **5**, 207.
4. Csonka, F. A.: A colorimetric method for the determination of acetone in urine. *J. Biol. Chem.*, 1916, **27**, 209.
5. Denigès, G.: Sur les functions organiques pouvant se combiner au sulfate mercurique. Cas des acétones. *Compt. rend. Acad. Sci.*, 1898, **126**, 1868. Combinaison, recherche, et dosage de d'acétone ordinaire avec le sulfate mercurique. *Compt. rend. Acad. Sci.*, 1898, **127**, 963.
6. Fabinyi, R.: Verfahren zur Darstellung eines neuen Seidenfarbstoffes. *Chem. Centralblatt*, 1900, **2**, 302.
7. Folin, O.: On the separate determination of acetone and diacetic acid in diabetic urine. *J. Biol. Chem.*; 1907, **3**, 177.
8. Folin, O., and Denis, W.: Turbidity methods for the determination of acetone, acetoacetic acid and β-oxybutyric acid in urine. *J. Biol. Chem.*, 1914, **18**, 263.
9. Frommer, V.: Neue Reaktion zum Nachweis von Aceton, samt Bemerkungen über Acetonurie. *Berl. klin. Woch.*, 1905, **42**, 1008.
10. Hubbard, R. S.: Determination of the acetone bodies in urine. *J. Biol. Chem.*, 1921, **49**, 357. Determination of the acetone bodies in blood. *Ibid.*, p. 375.
11. Hurtley, W. H.: The four carbon atom acids of diabetic urine. *Quart. J. Med.*, 1915–16, **9**, 301.
12. Marriott, W. McK.: The determination of β-oxybutyric acid in blood and tissues. *J. Biol. Chem.*, 1913–14, **16**, 293.
13. Messinger, O.: Titrimetrische Bestimmung von Aceton in Methylalkohol. *Ber deutsch. Chem. Ges.*, 1888, **21**, 366.
14. Personne, M. J.: Mémoire sur le dosage du mercure par les volumes à l'aide de liqueurs titrées. *Compt. rend. Acad. Sci.*, 1863, **56**, 951.
15. Scott-Wilson, H.: A method for estimating acetone in liquids. *J. Physiol.*, 1911, **42**, 444.
16. Shaffer, P. A.: A method for quantitative determination of β-oxybutyric acid in urine. *J. Biol. Chem.*, 1908–09, **5**, 211.
17. Shaffer, P. A.: Antiketogenesis: its mechanism and significance. *Medicine*, 1923, **2**, 375.

18. Short, J. J.: The formation of acetone bodies following ether anesthesis and their relation to the plasma bicarbonate. *J. Biol. Chem.*, 1920, **41**, 503.
19. Van Slyke, D. D.: Studies of acidosis. VII. The determination of β-hydroxybutyric acid, acetoacetic acid, and acetone in urine. *J. Biol. Chem.*, 1917, **32**, 455.
20. Van Slyke, D. D., and Fitz, R.: Studies of acidosis. VIII. The determination of β-hydroxybutyric acid, acetoacetic acid, and acetone in blood. *J. Biol. Chem.*, 1917, **32**, 495; 1919, **39**, 23.

CHAPTER XVIII

Total Organic Acids

TITRATION OF TOTAL ORGANIC ACIDS IN URINE. VAN SLYKE AND PALMER (5, 8)

In 1920 Van Slyke and Palmer (8) proposed a method for the titration of the total organic acid in urine, based on the following principles:

1. Relatively little strong mineral acid is required to change the pH of a water solution from 8 to 2.7 if the only electrolytes present are alkali salts of strong acids, such as sulfates and chlorides.
2. If the salt of a weak acid, of pK′ 4.2 to 6.2, is present the buffer effect is such that the addition of nearly a full molecule of hydrochloric acid for each equivalent of such salt is required to lower the pH from 8 to 2.7.
3. The organic acids known to occur in quantitatively significant amounts in normal and pathological urines belong to the class of weak acids whose salts behave in the manner described in 2. The only weak mineral acids found in significant quantities in urine are phosphoric and carbonic acids.
4. Some weak bases form buffer salts which titrate like those of weak acids. Creatinine is titrated almost quantitatively and creatine to the extent of 60 per cent when the pH is changed from 8 to 2.7. Aside from traces of amino acids, these appear to be the only such bases present in considerable amount in human urine.

The method in its original form consists simply of shaking the urine with an excess of calcium hydroxide to remove carbonate and phosphate, bringing the filtrate to pH 8 with phenolphthalein, and titrating to pH 2.7 with 0.2 N hydrochloric acid, tropeolin OO serving as indicator. Certain types of urines have been found for which this procedure must be modified. These cases will be discussed after the general method.

Reagents

Calcium hydroxide, powdered.

Hydrochloric acid, concentrated.

Hydrochloric acid dilute, 1 volume of concentrated hydrochloric acid made up to 10 volumes with water.

Hydrochloric acid, 0.2 N.

One per cent solution of phenolphthalein.

0.02 per cent solution of tropeolin OO.

0.04 per cent solution of bromophenol blue, for preparation see chapter on determination of pH.

Procedure

Place about 100 cc. of urine in a beaker. If the urine is albumin-free and is acid to litmus, add 2 grams of finely powdered calcium hydroxide and mix thoroughly. Let the mixture stand about 15 minutes, with occasional stirring. Then pass it through a dry folded filter.

Twenty-five cubic centimeters of the filtrate are measured into a large Pyrex glass test tube of 125 to 150-cc. capacity and 25 or 30-mm. diameter, with a mark at 60 cc. Add 0.5 cc. of phenolphthalein and titrate with 0.2 N hydrochloric acid from a burette until the pink color just disappears from the solution. This brings the solution to a pH of 8.0.

Five cubic centimeters of 0.02 per cent tropeolin OO are now added gradually while the tube is shaken constantly to insure mixture of its contents. If the shaking is omitted some of the dye may be precipitated. The solution is then titrated with 0.2 N hydrochloric acid until it attains a red color that matches that produced in a similar tube by 0.6 cc. of 0.2 N hydrochloric acid, 5 cc. of tropeolin OO solution and water to make 60 cc. As the end-point is approached enough water is added to the unknown to bring its volume to 60 cc.

In comparing the color of the titrated solution with that of the standard it is convenient during the titration to hold the two tubes side by side between the thumb and forefinger, the tube containing the urine being the one held nearer to the tips of the fingers, where it can be easily shaken as the acid is run in from the burette.

Modifications required in certain urines

In urines which are albuminous, which are alkaline to litmus, or in which the end-point with tropeolin OO fades, the procedure must be altered as follows.

1. ***Albuminous urines.*** Urine proteins act as buffers over the pH range of the titration, and therefore if present would titrate as organic acids. Palmer (5) found that small amounts are removed by the calcium hydroxide treatment. If much is present, however, it must be removed by acidifying by adding 1 to 3 drops of concentrated HCl to about 100 cc. of urine, bringing to the boiling point, and filtering. The filtrate is treated with calcium hydroxide, etc., as above described.

2. ***Alkaline urines with high bicarbonate content.*** Palmer

(5) found that if bicarbonate was present in concentration greater than 60 millimolar simple shaking with calcium hydroxide failed to remove all of the carbonate. Such a bicarbonate content is found in alkaline urine of pH over 7.7 (2). In such urines the CO_2 content is sufficiently lowered by acidifying with a few drops of 10 per cent hydrochloric acid and shaking the sample vigorously until most of the liberated CO_2 has been driven off. Palmer (5) recommends that whenever the pH of the urine is over 7 (alkaline to litmus) this procedure should be applied, or that the filtrate after the calcium hydroxide treatment should be tested for carbonates. For this test 2 or 3 drops of 10 per cent calcium chloride solution may be added to 2 or 3 cc. of the filtrate in a test tube: a precipitate of $CaCO_3$ forms if all the carbonate has not been removed. If a precipitate is obtained a fresh sample of urine is acidified and shaken before it is treated with calcium hydroxide.

3. *Urines in which tropeolin OO fades when acidified.* In the filtrates from some urines the tropeolin OO fades after the acid end-point at pH 2.7 is reached. Palmer (5) noted that when such fading had occurred the upper part of the urine, in contact with the air, might in time turn red again, indicating that the fading was due to the action of some reducing agent in the urine. He recommended that in such urines bromophenol be substituted as the indicator. However, it does not give so sharp an endpoint as tropeolin OO, and its color is more affected by the urinary pigments. McClusky (4) therefore recommends adding 0.5 cc. of concentrated hydrochloric acid per 100 cc. of urine in such cases and drawing a current of air through the fluid for a half-hour. This treatment oxidizes the reducing substance, so that tropeolin OO can be used, and also removes any carbonates which may be present. Calcium hydroxide is then added, and the analysis is completed in the usual manner.

As an alternative procedure the analysis can be carried through in the usual way, with acidifying and aerating, but with bromophenol instead of tropeolin OO as indicator. Because of the difficulty in judging the end-point, it is desirable to use a comparator box to compare the titrated tube with the standard as the end-point is approached.

It is advisable as a matter of routine to let the tubes stand fifteen minutes after they are titrated, and then to note by comparing them again with the standards whether fading in any has occurred. In cases where it has, the titration is repeated, either after acidifying and aerating, or with bromophenol blue as indicator in place of tropeolin OO.

4. Preliminary removal of creatine and creatine by adsorption with kaolin. Greenwald (3) has found that if urine is acidified and shaken with Lloyd's reagent all the creatinine and most of the creatine are removed, while lactic, acetic, formic, and beta-hydroxybutyric acids are not removed. With normal urines the treatment makes the results lower than by the regular Van Slyke-Palmer technique even with a correction subtracted for the creatinine. It therefore appears probable that urines contain appreciable amounts of basic buffers other than creatine, creatinine, and amino acids which are titrated over the pH range 8.0 to 2.7. The use of Greenwald's kaolin treatment will doubtless be found advantageous when the most exact results are desired. When relatively gross changes in organic acid excretion are being observed, as in diabetic ketosis, however, this refinement is unnecessary. The technique of the treatment is as follows:

Mix 100 cc. of urine with 100 cc. of 1 N sulfuric acid and 30 grams of Lloyd's reagent. The mixture is shaken thoroughly and filtered. The filtrate is treated with excess of calcium hydroxide, filtered again, and 50-cc. portions are titrated from pH 8 to 2.7, as directed for the usual method. The blank is somewhat higher than with the original method.

Calculation

From the volume of 0.2 N HCl used to titrate from the end-point of phenolphthalein to that of tropeolin OO, the amount, usually 0.7 cc., is subtracted which is utilized in a similar titration of a blank in which water is substituted for the urine.

In order to calculate the result in terms of milli-equivalents, or cubic centimeters of 1 N, organic acid per liter of urine, the number representing the corrected cubic centimeters of 0.2 N HCl used in the titration is multiplied by 8 (by $\frac{1000}{25} = 40$ in order to calculate from 25-cc. sample to 1000 cc. of urine, and by 0.2 in order to change from terms of 0.2 to 1 N).

Correction for creatinine. A molecular solution of creatinine (113.2 mg. per cubic centimeter) titrates in the above determination as 1 N solution of organic acid. Therefore, in order to correct for the creatinine, the cubic centimeters of 1 N organic acid per liter calculated from the above titration are diminished by

$$\frac{\text{Milligrams of creatinine per liter urine}}{113.2} \text{ or by } \frac{\text{milligrams creatinine N per liter urine}}{42}$$

Example

0.2 N HCl used in titration	7.6 cc.
Correction found in blank analysis	0.7 cc.
Corrected titration result	6.9 cc.

Milli-equivalents organic acid per l. = 8 × 6.9 = 55.2 uncorrected for creatinine.

Creatinine N content of urine = 500 mg. per liter.

Correction for creatinine $= \frac{500}{42.2} = 11.9$ m.-Eq.

Organic acid content corrected for creatinine = 55.2 − 11.9 = 43.2 m-Eq. per l.

The total twenty-four-hour organic acid excretion in a number of normal subjects examined by Van Slyke and Palmer (8) was found to be 0.6 to 1.0 cc. of 1 N acid per kilo body weight uncorrected for creatinine, or 0.2 cc. per kilo less when corrected for creatinine.

The chief clinical application found by the method has been its use as a simple means of following changes in the excretion of the acetone bodies in diabetic ketosis. For this purpose it is not necessary to correct for creatinine.

Greenwald (3) has found in the urines of some pneumonia patients abnormally large amounts of unidentified organic buffer substances which are titrated like organic acids in the Van Slyke-Palmer method, but which behave in a manner to suggest that they may be like creatinine, basic rather than acid buffers. They are partially precipitated by phosphotungstic acid and are entirely adsorbed by kaolin.

ORGANIC ACIDS IN BLOOD

For organic acids in blood there exists no method of such simplicity as Van Slyke and Palmer's titration for urine. Titration of blood filtrate with indicators does not give satisfactory results. The end-points are not sharp and the results appear obviously fallacious. Perlzweig and Delrue (6) have used the quinhydrone electrode to apply the same principle of titration between two end-points (pH 8 and 2.3) to the filtrate obtained after precipitating the blood proteins and treating the filtrate with lime and copper sulfate. They report values of 5 to 8 milli-equivalents of organic buffer of pK′ about 3.8 in normal blood. The method has not received sufficient application, however, to indicate its general utility. There appears to be no evidence concerning what part of the buffer effect measured is due to amino acids and other bases.

The alternative procedure that has been in use has been an indirect estimation of the undetermined anions in the serum. The total mineral base is determined, and from it are subtracted the equivalents of base bound by Cl, HCO_3, SO_4, PO_4, and serum protein. At pH 7.4 each mole of PO_4 binds 1.8

equivalents of alkali, hence the molar concentration of PO_4 is multiplied by 1.8 to estimate the equivalent concentrations. The equivalents of base bound by the proteins are estimated from the data of Van Slyke, Hastings, Hiller, and Sendroy (7). According to their data the base binding powers of the albumin and globulin are indicated by the following formulae:

$$\text{Milli-equivalents base bound per gram albumin nitrogen} = 0.78\ (pH_s - 5.16)$$

$$\text{Milli-equivalents base bound per gram globulin nitrogen} = 0.48\ (pH_s - 4.89)$$

$$\text{Milli-equivalents base bound per gram total serum protein nitrogen} = 0.66\ (pH_s - 5.08)$$

The equation for base bound by total serum proteins is calculated for the condition that the ratio of albumin to globulin is 1.6:1, and loses in accuracy as the ratio deviates from this value.

To express the formulae in terms of grams protein instead of nitrogen the constants 0.78, 0.48, and 0.66 are divided by 6.25, and thereby replaced by 0.125, 0.077 and 0.106 respectively. At pH 7.4, therefore, one calculates that each gram of albumin binds 0.28 milli-equivalents of base, each gram of globulin 0.193 milli-equivalents, and each gram of the total protein 0.246 milli-equivalents.

One therefore estimates the undertermined anions in serum or plasma at pH 7.4 as:

$$\text{Total base} - (Cl + HCO_3 + 1.8\ PO_4 + 2\ SO_4 + 0.28\ \mathit{Alb} + 0.193\ \mathit{Glob}).$$

The symbols, Cl, HCO_3, PO_4, and SO_4 represent millimols per liter. *Alb* indicates grams of albumin per liter, and *Glob* grams of globulin. In place of (0.28 *Alb* + 0.194 *Glob*) one may substitute as an approximation 0.246 *Prot*, where *Prot* is total protein.

From the most recent data of Atchley and Benedict (1), it appears that the normal blood serum contains organic acids in slight amount. The values of these authors for "undetermined anions" calculated in the above manner (but omitting the sulfate, present only in traces) for normal serum were found to vary from 3.8 to −3.0 milli-equivalents. The average of 15 analyses was, for the anions in milli-equivalents per liter; Cl, 103.5; HCO_3, 29.0; PO_4 (×1.8) 1.8, protein, 17.6; total anions, 151.8. Average total base was 151.9, average undetermined anions practically zero.

In pathological blood the SO_4 can not always be neglected in the calculation. In cases of renal retention Atchley and Benedict (1) found serum SO_4 as high as 7 milli-equivalents per liter.

BIBLIOGRAPHY

1. Atchley, D. W., and Benedict, E. M.: Serum electrolyte studies in normal and pathological conditions: pneumonia, renal edema, cardiac edema, uremic and diabetic acidosis. *J. Clin. Inv.*, 1930, **9**, 265.
2. Gamble, J. L.: Carbonic acid and bicarbonate in urine. *J. Biol. Chem.*, 1922, **51**, 295.
3. Greenwald, I.: Studies on metabolism in pneumonia. I. The excretion of organic acid and a method for its determination. *J. Biol. Chem.*, 1930, **85**, 447.
4. McCluskey, K. L.: The fading of tropeolin OO in the titration of organic acids in urine. *J. Biol. Chem.*, 1931, **90**, 197.
5. Palmer, W. W.: The titration of organic acids in urine. *J. Biol. Chem.*, 1926, **68**, 245.
6. Perlzweig, W. A., and Delrue, G.: Method for electrometric titration of organic acids of the blood. *Proc. Soc. Exp. Biol. Med.*, 1928, **25**, 548.
7. Van Slyke, D. D.; Hastings, A. B., Hiller, A., and Sendroy, J., Jr.: Studies of gas and electrolyte equilibria in blood. XIV. The amounts of alkali bound by serum albumin and globulin. *J. Biol. Chem.*, 1928, **79**, 769.
8. Van Slyke, D. D., and Palmer, W. W.: Studies of acidosis. XVI. The titration of organic acids in urine. *J. Biol. Chem.*, 1920, **41**, 567.

CHAPTER XIX

Phenols

DISCUSSION

Earlier investigators employed for the determination of phenols iodometric titration methods that required large amounts of material (5) (as much as 500 cc. of urine). In 1915 Folin and Denis (5) proposed a colorimetric procedure for the analysis of urine, based on the ability of phenols to reduce W^{vi} and Mo^{vi} to colored products of lower valence, which was clinically practical. Uric acid reduces in the same way, and hence had to be removed by precipitation with silver lactate. Tisdall (13) claimed that the method was not specific for phenols and presented another which he believed more accurate and which gives values only about half as great as those obtained by the Folin and Denis method. Folin and Ciocalteu (6) have apparently met the criticism by modifying the preparation of the phenol reagent so that it is more specific.

Adaptations of the Folin and Denis method to the analysis of blood have been made by Benedict and Theis (3), Pelkan (10), and Rakestraw (11). The principles of all these adaptations are the same: the removal of uric acid and proteins from blood to furnish a filtrate suitable for treatment with the Folin phenol reagent. The methods employed for the removal of uric acid are various. Theis and Benedict (12) later substituted *p*-nitroaniline for the Folin and Denis phenol reagent. *p*-nitroaniline has the advantage that it is not affected by uric acid, which therefore need not be removed.

Folin and Denis (5) have adapted their method to the analysis of feces. Hanke and Koessler (8) have devised other methods for the separate determination of tyrosine, tyramine and other phenols in biological materials.

Becher (1, 2) has devised a method for determination of free and conjugated phenols that depends on the use of Millon's reagent, which he and his associates have employed for clinical studies.

We shall describe for urine Folin and Denis' method with the improved Folin and Ciocalteu reagent. For blood the *p*-nitroaniline method of Theis and Benedict is described, and appears to deserve preference because it does not require removal of uric acid. When urine and blood analyses are made together, it is convenient to use the same reagent for both. Hence a blood method (Rakestraw's) in which the Folin reagent is applied is also described. *p*-nitroaniline has not been used for urine.

PHENOLS IN URINE. COLORIMETRIC MOLYBDITUNGSTATE METHOD OF FOLIN AND DENIS (5)

Reagents

Acid silver lactate. A 3 per cent solution of silver lactate in 3 per cent lactic acid (3.5 volume per cent of concentrated (85 per cent) lactic acid).

Colloidal iron solution. A 10 per cent solution.

Acid sodium chloride. A saturated solution of sodium chloride each liter of which contains 10 cc. of concentrated hydrochloric acid.

Phenol reagent of Folin and Ciocalteu (6). Dissolve 100 grams of sodium tungstate, $Na_2WO_4 \cdot 2H_2O$, and 25 grams of sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$ in 700 cc. of water in a 1500-cc. florence flask. Add 50 cc. of syrupy (85 per cent) phosphoric acid and 100 cc. of concentrated hydrochloric acid. Connect the flask with a reflux condenser by means of a cork or rubber stopper wrapped in tin foil. Boil the solution gently for ten hours. At the end of this time add 150 grams of lithium sulfate, 50 cc. of water and a few drops of liquid bromine. Boil the mixture without the condenser for about fifteen minutes to remove the excess bromine. Cool, dilute to 1 liter and filter. The finished reagent should have no greenish tint. It should be kept well protected from dust, because organic materials will gradually produce slight reductions.

Saturated sodium carbonate solution.

Standard phenol solution. Prepare a solution of phenol in 0.1 N hydrochloric acid which contains approximately 1 mg. of crystallized phenol per 1 cc. Tranfer 25 cc. of this solution to a 250-cc. flask, add 50 cc. of 0.1 N sodium hydroxide, and heat to 65°C. To the hot solution add 25 cc. of 0.1 N iodine solution; stopper the flask and let it stand at room temperature for thirty to forty minutes. Add 5 cc. of concentrated hydrochloric acid and titrate the excess of iodine with 0.1 N thiosulfate solution. Each cubic centimeter of 0.1 N iodine (cubic centimeters of iodine added—cubic centimeters of thiosulfate used in titration) corresponds to 1.567 mg. of phenol. On the basis of the titration dilute the phenol solution so that 1 cc. contains 0.1 mg. of phenol. (For preparation of standard iodine and thiosulfate solutions see pp. 32–34, chapter I.)

Concentrated hydrochloric acid.

Procedure

Removal of interfering substances. **To 10 cc. of undiluted urine or 20 cc. of very dilute urine, in a 50-cc. volumetric flask, add from 2 to 20 cc. of acid silver lactate, until no further precipitation occurs. Add a few drops of colloidal iron, shake the flask, dilute to the mark with**

water, shake again, and filter the contents through a dry filter. Transfer 25 cc. of the filtrate to another 50-cc. volumetric flask and add enough of the acid sodium chloride solution to precipitate all the silver. Fill the flask to the mark with water, mix the contents thoroughly and filter them through a dry filter.

Determination of free phenols: To 20 cc. of the filtrate in a 50-cc. volumetric flask add 5 cc. of the phosphotungstic-phosphomolybdic reagent and 15 cc. of saturated sodium carbonate solution. Dilute the solution to volume with luke warm (30° to 35°) water, mix thoroughly, and, after twenty minutes compare in a colorimeter with 5 cc. of the phenol standard, which has been treated with 10 cc. of phosphotungstic-phosphomolybdic reagent and 25 cc. of saturated sodium carbonate, diluted to 100 cc.

Total phenols (free and conjugated). To 20 cc. of the urine filtrate, in a large test tube, add 10 drops of concentrated hydrochloric acid; cover the tube with a small funnel, heat rapidly to boiling over a free flame and then place it in a boiling water-bath for ten minutes. At the end of this time remove the tube, cool and transfer its contents to a 100 cc. volumetric flask. Add 10 cc. of phosphotungstic-phosphomolybdic reagent, 25 cc. of saturated sodium carbonate, dilute the mixture to the mark with luke warm water (30° to 35°). Allow the tube to stand for 20 minutes and then compare in the colorimeter with a standard solution treated as described above.

Calculation

Filtrate equivalent to only one-fifth of the volume of urine originally taken is used for the final colorimetric comparison. The solution of free phenols is diluted, for colorimetric comparison, to one-half the volume of the standard, while the solution of total phenols is diluted to the same volume as the standard. The standard contains, in each case 0.5 mg. of phenol. Therefore,

$$\frac{1.25\,S}{V\,U} = \text{grams of free phenol per liter of urine.}$$

$$\frac{2.5\,S}{V\,U} = \text{grams of total phenol per liter of urine.}$$

S and U = colorimetric readings of standard and unknown respectively, and V is the volume of urine originally taken for analysis.

(Total phenol) − (free phenol) = conjugated phenol.

PHENOLS IN BLOOD. RAKESTRAW'S ADAPTATION OF THE COLORIMETRIC METHOD OF FOLIN AND DENIS (11)

This is an application of the Folin and Denis (5) urine method to the analysis of Folin-Wu tungstic acid blood filtrates. For the preliminary precipitation of uric acid the zinc method of Morris and Macleod is employed.

Reagents

Zinc chloride, a 2.5 per cent solution.

Sodium carbonate, a 10 per cent solution.

Sodium carbonate, a 20 per cent solution.

Phenol reagent of Folin and Ciocalteu, described in the urine method of Folin and Denis, above.[1] A portion of this is diluted with 3 volumes of water before using.

Sodium cyanide, a 5 per cent solution to each 100 cc. of which 3 or 4 drops of concentrated ammonia water are added. The solution should be kept in a bottle stoppered with a cotton plug to allow partial access of air to the solution.

Standard phenol solution, prepared as described in the urine method above. The stock solution is diluted as required so that 10 cc. contains 0.025 mg. of phenol.

Sodium hydroxide, a 20 per cent solution.

Procedure

Determination of free phenols. To 25 cc. of Folin-Wu tungstic acid blood filtrate (see precipitation of proteins, p. 65) add 1 cc. of 2.5 per cent zinc chloride and 1 cc. of 10 per cent sodium carbonate solution. After stirring the mixture, let it stand for one hour. Centrifuge and then filter off the supernatant fluid. To 10 cc. of the filtrate add 0.5 cc. of the diluted phenol reagent and 2 cc. of 20 per cent sodium carbonate solution. After mixing, allow the solution to stand thirty seconds. Then add 1 cc. of 5 per cent sodium cyanide (***this should be introduced from a burette, not a pipette, because of its poisonous character***). Immerse in boiling water for one and one-half minutes and then cool for at least three minutes in running water. Compare the color of the solution with a standard which has been subjected to the same treatment as the unknown.

[1] Rakestraw used Bell's modification of the Folin and Denis reagent. The modification of Folin and Ciocalteu appears to be at least as good.

Determination of total (free plus conjugated) phenols. To 10 cc. of the uric acid-free filtrate in a test tube, add 0.25 cc. of concentrated hydrochloric acid. Close the tube by means of rubber tubing and a screw-clamp pinch cock and heat it in a boiling water bath for ten minutes. Ten cubic centimeters of the standard solution are also treated with acid, but not heated. After they have been cooled, to both unknown and standard are added 0.5 cc. of 20 per cent sodium hydroxide, which nearly neutralizes the hydrochloric acid. Standard and unknown are then treated with phenol reagent, carbonate and cyanide as in the determination of free phenols and are compared in a colorimeter.

Calculation

$$\frac{100\ n\ S}{V\ U} = \text{milligrams of phenol per 100 cc. of blood.}$$

Where S and U = colorimetric readings of standard and unknown, respectively; n = the milligrams of phenol in the standard; and V = the cubic centimeters of blood represented by the quantity of filtrate taken for analysis.

With 10 cc. of uric acid-free filtrate (equivalent to $\frac{1}{2}\frac{0}{7}$ of a cubic centimeter of blood, and a standard containing 0.025 mg. of phenol, this becomes

$$\frac{6.75\ S}{U} = \text{milligrams of phenol per 100 cc. of blood.}$$

COLORIMETRIC *p*-NITROANILINE METHOD OF THEIS AND BENEDICT (12)

This method depends upon the reddish color produced by the reaction between phenol and diazotized *p*-nitroaniline base, described by Moir (9). This reagent is stated to be so insensitive to uric acid that preliminary removal of this substance is unnecessary.

Reagents

p-nitroaniline. Dissolve 1.5 grams of *p*-nitroaniline base in 500 cc. of water with 40 cc. of concentrated hydrochloric acid. When required for use a small quantity is mixed with 10 per cent sodium nitrite solution in the proportions, 25 cc. of *p*-nitroaniline solution to 0.75 cc. of sodium nitrite. This diazotized reagent does not keep longer than one day, so that the nitrite should be added only to such small quantities of the base as are required for immediate use.

Phenol standards prepared and standardized according to the method of

Folin and Denis, described in the method for analysis of urine, above. A dilute stock standard, 1 cc. of which contains 0.1 mg. of phenol, is made every few weeks and from this a 40-fold dilution (to 10 cc. = 0.025 mg. of phenol) is made daily.

One per cent gum acacia solution.

Fifty per cent sodium acetate solution.

Twenty per cent sodium carbonate solution.

Concentrated hydrochloric acid.

Procedure

Precipitation of proteins. The blood is precipitated with tungstic acid as in the Folin-Wu procedure (see precipitation of proteins, p. 65) except that the dilution is made 1:5 instead of 1:10 by using only 2 volumes of water instead of 7. Lake 1 part of tlood with 2 parts of water and add 1 part of $\frac{2}{3}$ N sulfuric acid slowly, with shaking. Finally introduce 1 cc. of 10 per cent sodium tungstate solution and mix the whole thoroughly. Filter.

Determination of free phenols. To 10 cc. of the filtrate add 1 cc. of 1 per cent gum acacia, 1 cc. of 50 per cent sodium acetate and 1 cc. of the diazotized *p*-nitroaniline reagent. After one minute add 2 cc. of 20 per cent sodium carbonate. Ten cubic centimeters of standard phenol solution, containing 0.025 mg. of phenol, are simultaneously treated in a similar manner. Standard and unknown are compared in the colorimeter.

Determination of total phenols. Ten cubic centimeters of the 1:5 blood filtrate are placed in a test tube with 0.25 cc. of concentrated hydrochloric acid and heated for ten minutes in a boiling water bath. The solution is cooled and the added acid neutralized with sodium hydroxide. The same amounts of acid and alkali are also added to the standard and to 10 cc. of unheated filtrate in which free phenols are to be determined. Phenols in the hydrolyzed and unhydrolyzed filtrates are then determined by the procedure for free phenols described above.

Calculation

$$\frac{100\ n\ S}{V\ U} = \text{milligrams of phenol in 100 cc. of blood.}$$

S and U = colorimetric readings of standard and unknown solutions, respectively, n = milligrams of phenol in the standard and V the cubic centimeters of blood represented by the quantity of filtrate taken for analysis.

With 10 cc. of filtrate (equivalent to 2 cc. of blood) and a standard containing 0.025 mg. of phenol, this becomes

$$\frac{1.25\ S}{U} = \text{milligrams of phenol per 100 cc. of blood.}$$

(Total phenol) − (free phenol) = conjugated phenol.

BIBLIOGRAPHY

1. BECHER, E.: Über das Vorkommen von aromatischen Oxysäuren und Phenolen im enteiweissten Blut und über die Bedeutung derselben bei echter Urämie. *D Arch. klin. Med.*, 1924, **145**, 333.
2. BECHER, E.; LITZNER, S., AND TÄGLICH, W.: Der Phenolgehalt des Blutes unter normalen und pathologischen Verhältnissen. I. Eine Bestimmungsmethode von freiem und gubundenem Phenol im Blut für klinische Zwecke. *Z. klin. Med.*, 1926, **104**, 182.
3. BENEDICT, S. R., AND THEIS, R. C.: The colorimetric determination of phenols in the blood. *J. Biol. Chem.*, 1918, **36**, 95.
4. CHAPIN, R. M.: The determination of cresol by the phenol reagent of Folin and Denis. *J. Biol. Chem.*, 1921, **47**, 309.
5. FOLIN, O., AND DENIS, W.: A colorimetric method for the determination of phenol (and phenol derivatives) in urine. *J. Biol. Chem.*, 1915, **22**, 305.
6. FOLIN, O., AND CIOCALTEU, V.: On tyrosine and trytophane determinations in proteins. *J. Biol. Chem.*, 1927, **73**, 627.
7. GOIFFON, R., AND NEPVEUX, F.: Appréciation colorimétrique des phénols urinaires. *Compt. rend. Soc., biol.*, 1923, **89**, 1213.
8. HANKE, M. T., AND KOESSLER, K. K.: Studies on proteinogenous amines. XIV. A microchemical colorimetric method for estimating tyrosine, tyramine and other phenols. *J. Biol. Chem.*, 1922, **50**, 235.

   XV. A quantitative method for the separation and estimation of phenols including phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine and tyramine. *J. Biol. Chem.*, 1922, **50**, 271.
9. MOIR, J.: *J. South African Inst.*, 1922, **5**, 8. (Cited by Theis and Benedict (12).)
10. PELKAN, K. F.: The physiology of the phenols. I. A quantitative method for the determination of phenols in the blood. *J. Biol. Chem.*, 1922, **50**, 491.
11. RAKESTRAW, N. W.: A quantitative method for the determination of phenols in blood. *J. Biol. Chem.*, 1923, **56**, 109.
12. THEIS, R. C., AND BENEDICT, S. R.: The determination of phenols in the blood. *J. Biol. Chem.*, 1924, **61**, 67.
13. TISDALL, F. F.: Estimation of the phenolic substances in urine. *J. Biol. Chem.*, 1920, **44**, 409.

CHAPTER XX

Hemoglobin and Its Derivatives

DISCUSSION

By total hemoglobin is meant the sum of all the forms of hemoglobin existing in the blood. Normally only reduced and oxygenated hemoglobin are present in significant amounts, and the sum of these two is customarily "hemoglobin content" of the blood. Occasionally carboxyhemoglobin or methemoglobin also is present. They do not interfere with the total hemoglobin methods that involve transformation of the hemoglobin into carboxyhemoglobin or methemoglobin respectively, or that depend upon determination of the iron content of blood.

For determination of the total hemoglobin in blood the most accurate method is ordinarily to saturate the blood with air or carbon monoxide, so that all the Hb present is changed to HbO_2 or HbCO, and to determine the O_2 or CO content. The "oxygen capacity" method was introduced by Haldane and Smith (11) in 1897; it became the standard by which other procedures were controlled. The average oxygen capacity of 18.5 cc. of O_2 per 100 cc. of blood found by Haldane and Smith (10) from analyses of the blood of several adults was for some time accepted as "100 per cent" of normal in clinical methods. More complete studies, the results of which are summarized in table 57, however, have shown that normal oxygen capacity varies greatly with age and sex, averaging, 20.7 volumes per cent for men, 19.0 for women and from 15.5 to 32 for children, depending upon age.

The greater rapidity of colorimetric methods and the minute amounts of blood with which they can be carried out has caused them to be generally preferred for routine clinical work to the more precise but less rapid gasometric procedures. Hemoglobin was first estimated colorimetrically in 1878 by Gowers (8), who merely diluted the blood and compared it with artificial standards of gelatin colored with picrocarmine. Oxyhemoglobin, however, was soon found, because of its instability and the nature of the yellowish-red color, to be a less satisfactory form of hemoglobin for colorimetric determination than some of the other derivatives. Carboxyhemoglobin (11, 13, 19), acid hematin (4, 9, 18), alkali hematin (33), cyanhemoglobin (25, 33) and methemoglobin (33) all have been utilized.

To match the colors of these hemoglobin derivatives the literature has contained a succession of artificial color standards, some in the form of solu-

tions (7, 12, 15, 18), others as colored glass (5, 6, 20, 16, 17). None has been entirely satisfactory because none has shown exactly the same light absorption throughout the visible spectrum as the hemoglobin solution with which it was to be matched. The standard prepared by most careful photometric work has apparently been that of Newcomer (16), who obtained a yellow glass with nearly the same absorption as acid hematin in all parts of the visible spectrum. The most satisfactory standards, so far as color is concerned, have remained those made from blood itself.

TABLE 57

NORMAL HEMOGLOBIN CONTENT OF HUMAN BLOOD. EFFECTS OF AGE AND SEX*

	OXYGEN CAPACITY; VOLUME PER CENT	
	Males	Females
1 day	32.0 ±4	32.0 ±4
2 to 3 days	29.0 ±4	29.0 ±4
4 to 8 days	26.0 ±4	26.0 ±4
9 to 13 days	23.0 ±4	23.0 ±4
2 to 8 weeks	20.5 ±4	20.5 ±4
3 to 5 months	17.5 ±3	17.5 ±3
6 to 11 months	16.0 ±3	16.0 ±3
1 to 2 years	15.5 ±2	15.5 ±2
3 to 5 years	16.4 ±2	16.4 ±2
6 to 10 years	17.3 ±2	17.3 ±2
11 to 15 years	18.0 ±2	18.0 ±2
16 to 60 years	*20.7 ±2*	*19.0 ±2*
60 to 70 years	19.9 ±2	19.0 ±2
70+ years	19.0 ±2	18.7 ±2

* See hemoglobin chapter of volume I

Most standard solutions prepared from blood, however, have shown the drawback of limited stability. Two changes cause their deterioration, alteration of color and development of cloudiness. The cloudiness was prevented by Palmer (19) by making his standard carboxyhemoglobin solution alkaline with ammonia, and Wu (33) has recently demonstrated the desirability of alkalinizing also standards prepared with cyanhemoglobin, methemoglobin, and acid hematin. Terrill (27) prepared acid hematin in the form of a dry powder which was permanent, and from which standard solutions could be made by weight.

Acid hematin, introduced by Sahli (22), is the derivative that has probably found most favor in the past, presumably because of the ease of its preparation, which requires only dilution of blood with 0.1 N hydrochloric

acid. It has been tested with approval by Newcomer (17), Cohen and Smith (4), Robscheit (21) and Senty (24), and undoubtedly gives fair approximation to the correct values in most blood, when the standards are prepared from similar bloods. Berczeller (2), however, found that the color developed is partly dependent on the plasma. A given suspension of sheep cells mixed with plasma from different sheep gave colors varying by 10 per cent or more from each other. Wu (33) also found that the color of blood diluted with acid, as in the acid hematin method, was much affected by non-hemoglobin substances in both plasma and cell stroma. Purified hemoglobin dissolved in plasma gave a 20 per cent higher reading than when dissolved in saline solution, and red cells that had merely been washed gave a lower color reading when suspended in plasma than in saline. These peculiar effects disappeared when the acidified hemoglobin and blood solutions were made alkaline before the colors were read. Wu attributed the anomalies of the acid solutions to the fact that in them the hemoglobin derivatives were in colloidal suspension rather than true solution, and the shade and intensity of color were influenced by factors affecting the state of dispersion. Wu found that better readings could be made also with methemoglobin and cyanhemoglobin when the solutions were clarified by alkali and confirmed the satisfactory character of Palmer's ammoniacal carboxyhemoglobin method. The total hemoglobin can be determined accurately after transformation into hematin, methemoglobin, or cyanhemoglobin, in alkaline solutions, even in blood that contains methemoglobin. When the readings are made with acid hematin, the presence of methemoglobin makes accurate readings impossible. Wu preferred cyanhemoglobin for colorimetric readings, although he obtained correct results with the other three alkaline solutions.

Other principles that have been employed for the determination of hemoglobin are interesting for special studies rather than for clinical use. Spectrophotometric analysis has been used by Williamson (30) and others; refractometry by Howard (14) and Stoddard and Adair (26). Wolter (31), Berman (1), Brown (3) and Wong (32) determined the iron content of blood as a measure of the total hemoglobin.[1]

[1] Mention must be made of the method of Tallquist (*Arch. gen. de. med.* n.s., 1900, 3, 421), which has perhaps been more extensively used for clinical purposes than any other one method. It depends on the comparison of the color of a drop of blood, absorbed on a piece of filter paper, with a colored chart. Although obviously only approximate, Tallquist's method is the simplest and most generally applicable means for the rapid estimation of hemoglobin and does not deserve the entire measure of disrepute which it has received. In order to obtain proper results certain precautions which are usually neglected must be

The standard gasometric oxygen and carbon monoxide capacity methods are described in the chapter on gasometric methods. Here we shall describe the Palmer carboxyhemoglobin method, the Cohen-Smith acid hematin determination with Wu's alkaline modification, the acid hematin procedure for use with Newcomer's glass standards, the Stadie-Wu cyanhemoglobin method, and Wong's method based on colorimetric determination of the iron content of blood.

Choice of Methods

For accuracy the gasometric methods, with an error of only 0.5 per cent, stand in a class above the colorimetric methods, which under the best conditions involve analytical errors of ± 2 or 3 per cent, which may be further increased by inaccuracy in standards, manipulation, or colorimetry. The colorimetric methods have an advantage, however, in that they can be carried through with great speed, and require only 0.02 to 0.20 cc. of blood.

For combined simplicity and accuracy among colorimetric procedures the Palmer carboxyhemoglobin method seems to claim preference when a satisfactory illuminating gas is available. Some gas is said to contain sufficient cyanogen to disturb the determination, but we have not heard of such difficulties with American gas. The Palmer method has an advantage unique among colorimetric procedures in that the stock standard can at any time have its accuracy controlled by direct gasometric analysis (28). The Stadie-Wu cyanhemoglobin gives an equally beautiful color to read and may be used when a satisfactory source of carbon monoxide gas is not available. The acid hematin methods are more subject to error, but are described because of their simplicity, and the fact that the Newcomer glass standard makes them available in laboratories that can not prepare their own standards from blood of determined oxygen capacity.

For a laboratory where only an occasional hemoglobin determination is required it will not be desirable to prepare and control standard blood solu-

---

observed. When blood is first taken up by the filter paper and before it is completely absorbed it presents a shining surface that makes color comparison impossible; when the blood begins to dry, on the other hand, it very rapidly assumes a brownish tinge that also interferes with color comparison. There is, then, only a brief interval during which the determination can be made. The length of this interval is proportional, within certain limits, to the size of the drop of blood taken. It is, therefore, essential to take sufficient blood to make a spot 1.5 to 2 cm. in diameter to obtain satisfactory results. Furthermore, during the reading the filter paper and the color scale must be backed by a solid white background to avoid the effects of shadows behind the filter paper, which is not entirely opaque.

tions for colorimetric analyses. In this case the Newcomer method will be the one of choice for approximate analyses, and the gasometric oxygen or carbon monoxide capacity method for accurate ones.

When a large number of determinations is routinely required the fifteen minutes per analysis required by the gasometric methods limits their use to cases where accuracy is especially important. For numerous routine analyses the colorimetric method of Palmer or of Stadie and Wu will be desirable.

When the blood contains abnormal forms of hemoglobin of uncertain nature, as in nitrobenzene poisoning (see volume I), the iron method of Wong can be used. In such cases other methods, both colorimetric and gasometric, may be inapplicable. Such cases are rarely encountered except in industrial poisoning by coal tar products.

As a refinement of micro analysis which has not yet been applied to clinical studies, the reader is referred to the ultra-micro method of Wu (34), in which the hemoglobin in 0.001 cc. of blood is determined by its action in catalysing the reaction between peroxide and benzidine.

To standardize the hemoglobin solutions used as standards for the colorimetric procedure Wong's iron method can be used as an alternative to the standard oxygen and carbon monoxide capacity determinations.

The calculations in this chapter are given in terms of volumes per cent oxygen capacity rather than in grams of hemoglobin. As pointed out in the discussion of "Total hemoglobin" in Volume I, no accurate data exist for calculating the exact weight of hemoglobin from gasometric or colorimetric analyses.

Calculations of percentages of normal hemoglobin content are arranged to compare the hemoglobin content of a subject's blood with the average hemoglobin content of normal subjects of the same sex and age. As shown in table 57, the variations with sex and age are so great that the hitherto common practice of using a single standard for all subjects gives fallacious results.

TOTAL HEMOGLOBIN. GASOMETRIC

Determinations of the oxygen or carbon monoxide binding capacity afford the most accurate estimation of total hemoglobin and are the standards on which the colorimetric methods are based. The O_2 and CO determinations are described on pages 263, 266, 337 and 341 in the chapter on gasometric methods. In the rare cases in which the blood contains methemoglobin and modifications of these methods described for that condition on page 340 are employed.

TOTAL HEMOGLOBIN. COLORIMETRIC CARBON MONOXIDE METHOD OF PALMER (19)

Reagents

Ammonia solution. Dilute 4 cc. of strong ammonia solution to a liter with water.

Stock standard hemoglobin solution

A quantity of *fresh* human blood or ox blood is defibrinated by stirring it with a rod. (If blood stands for a few hours significant amounts of methemoglobin may form.) After the defibrinated blood has been filtered through gauze its oxygen or carbon monoxide capacity is determined (see chapter on gasometric methods). The blood is then diluted with the ammonia solution to an oxygen capacity of 4 volumes per cent.[2] This hemoglobin solution is saturated in a dark glass bottle with carbon monoxide by bubbling through it preferably 2 or 3 volumes of CO gas (for its preparation see chapter on gasometric methods). As an alternative illuminating gas may be passed through for ten minutes, after the addition of a drop or more of caprylic alcohol to prevent foaming. After passage of the CO or illuminating gas the glass tube through which the gas was passed into the solution is withdrawn slowly and the bottle is immediately stoppered with a glass stopper or cork (rubber stoppers must not be used in contact with hemoglobin solutions). The stopper should be sealed with paraffin. The solution will keep for months in an ice-box protected from light *if kept saturated with pure carbon monoxide, but it is desirable to test it every month by determining its CO capacity* as described on p. 348 in the chapter on gasometric methods. Or, if CO gas is not available, the standard may be checked by comparing the results of a colorimetric hemoglobin determination with those of a gasometric oxygen capacity on any given sample of blood. As shown by Van Slyke and Hiller (28), *the bottle containing the stock hemoglobin solution must be refilled with carbon monoxide or illuminating gas every time the stopper is removed.* Otherwise air replaces the gas in the bottle and formation of methemoglobin begins, making the solution inaccurate as a colorimetric standard.

From this stock standard solution a dilute standard is prepared for routine use by diluting 50 cc. of the stock solution to 1010 cc. with the ammonia solution. The dilute standard is best kept in a dark glass or black painted aspirator bottle. Both openings of the aspirator bottle should be provided with corks fitted with glass tubes bearing glass stop-cocks. The corks must be sealed in with paraffin and the glass tubes should be long enough to project just through the corks into the bottle. When the solution is first made up the lower tube is connected to a gas jet which is opened. Both stop cocks are then opened and illuminating gas is permitted to bubble through the solution until the latter has become completely saturated and until the air in the bottle has been displaced. The

---

[2] The dilution may be calculated by the following equation:

$$\frac{HV_1}{4} = V_2$$

in which H = the oxygen capacity of the blood in volumes per cent.

V_1 = the volume of blood to be diluted.

V_2 = the volume to which it must be diluted to bring the oxygen capacity to 4 volumes per cent.

stop cocks are then closed and the gas jet is connected with the upper tube. The connecting tube between the gas jet and bottle should preferably be of glass or metal, since the air diffuses rather rapidly through rubber. With the upper stop cock and the gas jet continuously kept open, solution is withdrawn as desired from the lower end, the volume of withdrawn solution being automatically replaced by an equal volume of illuminating gas. By this means the liquid is kept from contact with air and complete combination of the hemoglobin with carbon monoxide is always assured. The dilute hemoglobin solution should be renewed every two weeks. The first indication of deterioration is the appearance of a brownish tinge in the cherry-red color characteristic of carbon monoxide hemoglobin.

Saturation of blood and standard solutions with illuminating gas should be carried out under a hood, removed from any open flame. If there is no hood available a funnel attached to a water vacuum pump may be used to carry off the gas.

Pipettes for measuring blood are calibrated *to contain* 0.05 cc. and 0.10 cc. ±0.0002 cc. They should hold when dry 0.671 and 1.348 grams of mercury, respectively (see p. 18–20 and figure 4). The pipettes can be made from millimeter bore glass tubing, the points being tapered on an emery wheel.

Procedure

Draw 0.05 cc. of blood into a blood pipette and thence transfer the sample to 5 cc. of the ammonia solution in a 12 by 120 mm. test tube. Rinse the blood pipette by drawing the ammonia solution into it two or three times. After bubbling illuminating gas through the mixture for thirty seconds, compare the color in a colorimeter with that of the dilute standard described above. If the blood contains less than 60 or 70 per cent of the normal hemoglobin content 0.1 cc. of blood should be taken instead of 0.05 cc.

Calculation

$\frac{20\,S}{U}$ = volumes per cent O_2 capacity when sample is 0.05 cubic centimeter of blood.

$10\,S$ = volumes per cent O_2 capacity when sample is 0.10 cubic centimeter of blood.

$$O_2\ Capacity \times \frac{100}{N} = \left\{\begin{array}{l}\text{hemoglobin content calculated as per cent of the average normal}\\ \quad\text{for the subject's age and sex.}\end{array}\right.$$

S and U represent readings of standard and unknown solutions respectively, N is the mean normal oxygen capacity for a person of the subject's age and sex. Values of N are given in table 57.

TOTAL HEMOGLOBIN. COLORIMETRIC ACID HEMATIN METHOD OF COHEN AND SMITH (4) MODIFIED BY WU (33) TO ALKALI HEMATIN

Preparation of standard

Defibrinate about 50 cc. of *freshly drawn* blood by stirring with a glass rod and strain the blood through a few layers of gauze. Determine the oxygen or carbon monoxide capacity of a sample of this blood (see chapter on gasometric methods). (The blood must be frequently stirred throughout the whole procedure to insure uniform mixture of cells and plasma.) Dilute the remainder of the blood with 0.1 N hydrochloric acid until the hemoglobin content corresponds to an oxygen capacity of 4 volumes per cent (see footnote on p. 666). Mix thoroughly and store in a glass-stoppered bottle in a cool dark place. This solution should last several months if kept free from molds. From this stock standard a dilute standard is made by diluting 5.0 cc. to 101 cc. with 0.1 N hydrochloric acid. The dilute standard, the hemoglobin content of which is equivalent to 0.2 volume per cent oxygen capacity, must be prepared fresh every week.

Procedure

By means of a pipette calibrated to *contain* that volume, measure 0.05 cc. of blood into 5 cc. of 0.1 N hydrochloric acid. Rinse the pipette by drawing acid up into it and expelling the liquid back into the solution from which it was drawn. If the blood contains very little hemoglobin 0.10 cc. should be taken. To develop the color, immerse the tube in water at 35 to 40° temperature for 10 minutes, or let stand at room temperature for 40 minutes. Add 0.5 cc. of 10 per cent sodium hydroxide solution. Compare in the colorimeter with standard solution, to which on the same day has been added one-tenth its volume of 10 per cent sodium hydroxide.

Wu's reason for adding the alkali is discussed on page 663.

Calculation

The calculation is the same as for Palmer's method above.

TOTAL HEMOGLOBIN. COLORIMETRIC ACID HEMATIN METHOD OF NEWCOMER WITH GLASS STANDARD (16)

This method has the advantage that the color standard is a glass plate, and therefore permanent. These plates, of approximately 1-mm. thickness, are obtainable from the Arthur H. Thomas Company, of Philadelphia, with the exact thickness engraved on each. The plates are made from one stock of glass which was found by Newcomer to have nearly the same light absorption as acid hematin.

The plate is placed in the light path of one cup of a Duboscq type of colorimeter and 5 cc. of water are placed in the cup. In the other cup

are placed 5 cc. of 0.1 N hydrochloric acid, and to it is added 0.02 cc. of blood from a capillary pipette. With the lower end of the prism on the side of the standard just immersed in the water, the colors are balanced by adjusting the prism in the blood solution.

Calculation

$$\frac{51\ d\ h}{U\left(100 - \frac{40}{T}\right)} = \text{volumes per cent oxygen capacity of blood.}$$

$$\frac{5100\ d\ h}{N\ U\left(100 - \frac{40}{T}\right)} = \text{per cent of normal hemoglobin for subject's age and sex.}$$

Where d = dilution (e.g., 251).

U = the observed thickness of the balancing hematin solution in millimeters.

h = the exact thickness of the glass in millimeters.

T = the time in minutes between dilution and reading.

N = mean normal oxygen capacity for subject's age and sex (see table 57).

The factor $\left(100 - \frac{40}{T}\right)$ corrects for the time factor in development of the acid hematin color.

When d = 251, as in the dilution technique described, and 30 to 40 minutes are allowed for the color to develop, the calculations simplify to

$$\frac{129\ h}{U} = \text{volumes per cent oxygen capacity.}$$

$$\frac{12900\ h}{N\ U} = \text{per cent of normal hemoglobin for subject's age and sex.}$$

TOTAL HEMOGLOBIN. COLORIMETRIC CYANHEMOGLOBIN METHOD OF STADIE (25) AS MODIFIED BY WU (33)

Reagents

Dilute ammonia. Four cubic centimeters of concentrated ammonia diluted in 100 cc. with water.

0.4 per cent potassium ferricyanide solution.

0.1 per cent potassium cyanide solution.

Standard solution.

Blood is standardized by the oxygen or carbon monoxide capacity method (see chapter on gasometric methods) and diluted with 4 per cent ammonia to an oxygen capacity of 4 volumes per cent as described in two preceding methods. Of this stock solution 50 cc. are placed in a 1 liter flask, are diluted with 200 cc. of 4 per cent ammonia, and 25 cc. of the 0.4 per cent potassium ferricyanide are added. After twenty minutes 25 cc. of the 0.1 per cent potassium cyanide are added, and the solution is diluted to 1 liter. The standard keeps a month at room temperature. The stock solution may be saturated with CO or illuminating gas, as in Palmer's method, to preserve it longer.

Procedure

Place 2 cc. of the 4 per cent ammonia solution in a flask or tube marked to contain 10 cc. Deliver 0.1 cc. of blood (or 0.2 cc. if blood is anemic) into this, washing out the pipette by drawing the ammonia solution up into it. Add 0.25 cc. of the ferricyanide to change the hemoglobin to methemoglobin. After twenty minutes add 0.25 cc. of the potassium cyanide solution to form cyanhemoglobin, and dilute to 10 cc. Read against the standard. (It is safer to measure the cyanide solution from a micro burette because of its poisonous character.)

Calculation

$$\frac{20\,S}{U} = \text{volumes per cent } O_2 \text{ capacity when sample is 0.1 cc. of blood.}$$

$$\frac{10\,S}{U} = \text{volumes per cent } O_2 \text{ capacity when sample is 0.2 cc. of blood.}$$

Rest of calculation as for Palmer method, above.

TOTAL HEMOGLOBIN. COLORIMETRIC DETERMINATION OF IRON IN BLOOD. (WONG (32))

The iron of the hemoglobin molecule is detached by the action of concentrated sulfuric acid, used as ordinarily in the preparation of acid hematoporphyrin, the decomposition being facilitated with the aid of potassium persulfate. The proteins are then precipitated by tungstic acid and filtered off. Since the whole process involves no boiling, the concentration of acid in the unknown can be controlled and made the same as in the standard for the production of color. The color fading is retarded by adding potassium persulfate to both unknown and standard.

Reagents

All the reagents except the standard must be iron-free as shown by blank tests.

1. *Concentrated sulfuric acid.*

2. *Sodium tungstate.* Ten per cent solution, as in the Folin-Wu method for precipitating blood proteins.

3. *Saturated potassium persulfate.* Introduce into a small glass-stoppered bottle about 7 grams of pure potassium persulfate and shake with 100 cc. of distilled water. The undissolved portion settles on the bottom and later in part dissolves to replace persulfate lost from the solution by the slow decomposition that occurs in the latter at room temperature.

4. *Potassium sulfocyanate.* Prepare approximately a 3 N solution by dissolving 146 grams of pure potassium sulfocyanate in distilled water to make 500 cc. Filter if necessary. Add 20 cc. of pure acetone to improve the keeping quality.

5. *Standard iron solution.* Weigh out accurately 0.861 grams of crystallized ferric ammonium sulfate and dissolve in about 50 cc. of distilled water. Add to the solution 20 cc. of 10 per cent iron-free sulfuric acid. Dilute with distilled water to 1 liter. Each cubic centimeter will contain 0.1 mg. of iron for use as a regular standard. To make weaker standards, dilute this standard solution accordingly.

Procedure

Transfer accurately 0.500 cc. of blood into a 50-cc. volumetric flask and introduce 2 cc. of Fe-free concentrated sulfuric acid. Whirl the flask to agitate the mixture for one or two minutes. Add 2 cc. of saturated potassium persulfate solution and shake. Dilute to about 25 cc. with distilled water, and add 2 cc. of 10 per cent sodium tungstic solution. Mix. Cool to room temperature under the tap, and then dilute to volume with distilled water. Stopper the flask and invert 2 or 3 times to effect thorough mixing. Filter through a dry filter paper into a clean, dry, receiving vessel. Pipette exactly 20 cc. of the clear filtrate into a large test tube graduated at 20 and 25 cc.

Measure into another similar test tube exactly 1 cc. of the standard solution containing 0.1 mg. of Fe per cubic centimeter. Add with a graduated 1-cc. pipette 0.8 cc. of Fe-free concentrated sulfuric acid and dilute to the 20 cc. mark with distilled water. Cool to room temperature under the tap. Now add to both the unknown and standard 1 cc. of saturated potassium persulfate and 4 cc. of 3 N potassium sulfocyanate solution. Insert clean rubber stoppers, mix and compare in a Duboscq colorimeter.

Calculation

The 20 cc. of filtrate taken represent 0.2 cc. of the original blood, and the quantity of standard solution used contains 0.1 mg. of Fe. Therefore,

$$\frac{S}{U} \times 50 = \text{milligrams of Fe per 100 cc. of blood.}$$

To obtain volumes per cent of O_2 capacity the figure representing mg. of Fe per 100 cc. is multiplied by $\frac{22.4}{55.9}$ or 0.4, since the amount of hemoglobin binding 1 mg. atom of Fe, or 55.9 mg., also binds 1 mg. molecule of O_2, or 22.4 cc. measured at 0°, 760 mm.

The calculation of hemoglobin in terms of O_2 capacity therefore becomes:

$$\frac{20\ S}{U} = \text{volumes per cent } O_2 \text{ capacity of blood.}$$

OXYGENATED AND REDUCED HEMOGLOBIN. GASOMETRIC

The blood is drawn from vein or artery under conditions completely preventing contact with air (see chapter on treatment of biological material, p. 53). The oxygen content is then determined as described in the chapter on gasometric methods. A portion of the blood is then used for determination of the total oxygen capacity or carbon monoxide capacity as described in that chapter. The oxygen saturation of the blood is then calculated as:

$$\text{Per cent oxygen saturation} = 100 \times \frac{\text{volumes per cent } O_2 \text{ bound by Hb in blood as drawn}}{\text{volumes per cent } O_2 \text{ or CO bound by Hb in saturated blood}}.$$

The oxygen bound by the hemoglobin in the drawn blood is slightly less than the total oxygen content because the blood contains a small amount of dissolved oxygen. This is subtracted from the total oxygen to give the oxygen combined as HbO_2. The amount to be subtracted can be estimated with sufficient accuracy from table 58. This table is calculated from the data of Bock charted in figure 52 of volume I. The dissolved O_2 is calculated as

$$\text{millimeters of } O_2 \text{ tension} \times \frac{0.8\ \alpha\ p}{7.6},$$

where α is the solubility coefficient of O_2 in water, p is the O_2 tension in millimeters of mercury. α is multiplied by 0.8 on the assumption that the solubility of O_2 in blood is proportional to the water content. The exactness of this assumption is uncertain, but the amounts of dissolved oxygen calculated with it are probably not far from correct. Winkler's value, 0.0236,

for α at 38° is used. Neglect to make the correction to observed blood O_2 contents in order to estimate O_2 bound to hemoglobin is apparently responsible for the reporting in the literature of some arterial oxygen saturations as high as 100 per cent, whereas 96 or 97 per cent appears to be the highest saturation possible in a subject breathing atmospheric air at sea level.

In place of determining the total hemoglobin by oxygen capacity gasometrically, one may, as a less accurate alternative, determine the total hemoglobin by one of the previously described colorimetric procedures.

TABLE 58

OXYGEN ESTIMATED HELD IN PHYSICAL SOLUTION BY BLOOD AT 38° AT DIFFERENT OXYGEN TENSIONS AND DEGREES OF HEMOGLOBIN SATURATION

SATURATION OF BLOOD Hb with O_2 AT pH_S 7.44	TENSION OF O_2	OXYGEN PHYSICALLY DISSOLVED IN BLOOD**
per cent	*mm. of Hg*	*vol. per cent*
0	0	0.00
10	7	0.02
20	12	0.03
30	16	0.04
40	20	0.05
50	24	0.06
60	30	0.08
70*	35*	0.09*
80	43	0.11
90	58	0.15
95†	75†	0.19†
100	ca. 120	0.30
100‡	139‡	0.35‡
100§	149§	0.40§

* Approximate for usual mixed venous blood.

† Approximate for usual arterial blood.

‡ Blood equilibrated *in vitro* at 760 mm. barometric pressure and 38° temperature with air containing CO_2 at 40-mm. tension.

§ Blood equilibrated *in vitro* at 760 mm. and 38° with air containing no added gas.

** Subtract from total blood O_2 content to obtain O_2 combined as HbO_2.

When oxygen saturations are determined, however, the conditions usually demand a maximum of accuracy for proper interpretation of the results, and it is desirable to adhere to gasometric procedures throughout.

CARBOXYHEMOGLOBIN. GASOMETRIC

For accurate estimation of the proportion of hemoglobin saturated in blood with carbon monoxide one must determine the CO content of the blood and the total CO binding capacity. These determinations are described in the chapter on gasometric methods, pages 328 and 341.

CARBOXYHEMOGLOBIN. APPROXIMATE COLORIMETRIC METHOD OF SAYERS AND YANTS (23)

A quick and approximate method that requires no special apparatus and can be used in the field in cases of suspected carbon monoxide poisoning has been devised by Sayers and Yant in the Bureau of Mines. It depends upon Wetzel's test, in which blood is treated with tannic acid. Normal blood thereby forms merely a gray suspension, while carbon monoxide blood forms a carmine red solution.

Reagents

Tannic acid solution. Two grams to 100 cc. of water.

Pyrogallol solution. Two grams to 100 cc. of water.

Tannic acid-pyrogallol mixture. Equal weights of the two substances ground together in a mortar.

Preparation of standards. Five cubic centimeters or more of normal blood are drawn and kept from clotting by the addition of 5 mg. of potassium citrate per cubic centimeter.

One-half of the blood is diluted to 10 volumes with water.

The hemoglobin in the other half is saturated with CO by rotating the blood about the walls of a 200-cc. bottle filled with air containing 3 to 5 per cent of carbon monoxide, and is then diluted to 10 volumes with water. For saturation a mixture of air and illuminating gas may be used. Of illuminating gas of the common "water gas" type, with about 35 per cent of CO, an 8 or 10-fold dilution with air gives the proper CO content. The blood is saturated with CO before it is diluted with distilled water, instead of after the dilution, in order to minimize the volume of CO gas physically dissolved in the final solution.

From these two solutions, one containing all the Hb in the form of HbO_2, the other in the form of HbCO, ten mixtures are made, each of 1 cc. volume. Amounts of 0.1, 0.2 cc., etc., of the HbCO solution are mixed with 0.9, 0.8 cc. etc., of the HbO_2 solution. The mixtures are placed in test tubes of thin clear glass, of approximately 15-mm. inside diameter.

Equal volumes of the tannic acid and pyrogallol solutions are now mixed, and of the mixture 1 cc. is added to each of the 10 standard solutions. Each tube is inverted twice to mix the contents, and is then sealed by pouring in about 2 cc. of melted paraffin in order to prevent oxygen of the air from acting on the HbCO-HbO_2 mixture. The paraffin is heated just enough to melt it and the test tubes are partially immersed in cold water while the paraffin is poured into them. After the paraffin has hardened the seal is

made permanent by placing a disk of paper over the paraffin in each tube and filling the remainder of the tube with sealing wax.

Standards thus made develop their full color in thirty minutes, and kept in a cool place will remain two weeks without sufficient change in color to interfere with the accuracy of the determination.

Procedure

A sample of 0.1 cc. of blood is drawn by ear or finger prick, and is diluted to 2.0 cc. with water. To the diluted blood approximately 40 mg. of the mixture of equal parts of tannic acid and pyrogallol is added at once. The contents of the tube are mixed by inverting the tube several times, but no more than is necessary, for escape of significant proportions of CO from the blood must be prevented. The tube is placed in a rack and allowed to stand for thirty minutes. It is then compared with the series of standard solutions, and the percentage of saturation of the hemoglobin with carbon monoxide is estimated by interpolation.

The observations are best made in broad daylight, but not in direct sunlight. The observer should stand with his back to the light, viewing the tubes by reflection, and should change their positions several times to note any difference due merely to unequal lighting effect. Observations may be made after several hours without serious loss of accuracy, although such delay is inadvisable.

METHEMOGLOBIN. GASOMETRIC

The chemical methods for determining methemoglobin thus far proposed are based upon gasometric determinations of the O_2 or CO combining capacity of the unchanged hemoglobin. Methemoglobin determination is accordingly treated entirely in the chapter on gasometric methods, page 353.

BIBLIOGRAPHY

1. BERMAN, L.: A rapid method for the determination of iron in small quantities of blood. *J. Biol. Chem.*, 1918, 35, 231.
2. BERCZELLER, L.: Über die colorimetrische Bestimmung des Hämoglobins als Säurehämatin. *Biochem. Z.*, 1918, 87, 23.
3. BROWN, A. L.: A new quantitative method for the determination of iron in the blood. *J. Am. Chem. Soc.*, 1922, 44, 423.
4. COHEN, B., AND SMITH, A. H.: The colorimetric determination of hemoglobin. A practical procedure. *J. Biol. Chem.*, 1919, 39, 489.
5. DARE, A.: A new hemoglobinometer for the examination of undiluted blood. *Philadelphia Med. J.*, 1900, 6, 557.

6. Dreyer, G.; Bazett, H. C., and Pierce, H. F.: Diurnal variations in the haemoglobin content of the blood. *Lancet*, London, 1920, **2**, 588.
7. Felton, L. D.: A substitute for acid hematin as the standard in Sahli's hemoglobinometer. *Johns Hopkins Hosp. Bull.*, 1923, **34**, 357.
8. Gowers, W. R.: Apparatus for the clinical estimation of the haemoglobin in the blood. *Trans. Clin. Soc., London. Lancet.* 1878, **2**, 882.
9. Haessler, H., and Newcomer, H.: A clinical hemoglobinometer. *Arch. Int. Med.*, 1916, **17**, 806.
10. Haldane, J.: The colorimetric determination of haemoglobin. *J. Physiol.*, 1900–01, **26**, 497.
11. Haldane, J. S., and Smith, J. L.: The mass and oxygen capacity of the blood in man. *J. Physiol.*, 1899–1900, **25**, 331.
12. Haskins, H. D.: A new permanent standard for Sahli's hemoglobinometer. *J. Biol. Chem.*, 1923, **57**, 111.
13. Hoppe-Seyler, F.: Verbesserte Methode der colorimetrischen Bestimmung des Blutfarbstoffgehaltes in Blut und in anderen Flüssigkeiten. *Z. physiol. Chem.*, 1892, **16**, 505.
14. Howard, F. H.: The determination of the refractivity of hemoglobin in solution. *J. Biol. Chem.*, 1920, **41**, 537.
15. Jacobson, V.: A new standard solution for Sahli's modification of Gower's hemoglobinometer. *J. Am. Med. Ass.*, 1919, **73**, 1282.
16. Newcomer, H. S.: Absorption spectra of acid hematin, oxyhemoglobin, and carbonmonoxide hemoglobin. A new hemoglobinometer. *J. Biol. Chem.*, 1919, **37**, 465.
17. Newcomer, H. S.: A new optical instrument for the determination of hemoglobin. *J. Biol. Chem.*, 1923, **55**, 569.
18. Osgood, E. E., and Haskins, H. D.: A new permanent standard for estimation of hemoglobin by the acid hematin method. *J. Biol. Chem.*, 1923, **57**, 107.
19. Palmer, W. W.: The colorimetric determination of hemoglobin. *J. Biol. Chem.*, 1918, **33**, 119.
20. Reichert, C.: Das Haemometer nach Professor Ed. Fleischl von Marxow, Wein, 1889.
21. Robscheit, F. S.: A comparative study of hemoglobin determination by various methods. *J. Biol. Chem.*, 1930, **41**, 209.
22. Sahli, H.: Diagnostic methods. Translation of Ed. 4, Philadelphia, 1905.
23. Sayers, R. R., and Yant, W. P.: Pyrotannic acid method for determination of carbon monoxide in blood and air. U. S. Bureau of Mines, 1925, Technical Paper 373, Washington, D. C.: *Analyst*, 1926, **51**, 99.
24. Senty, E. G.: A comparative study of various methods of hemoglobin determinations. *J. Lab. and Clin. Med.*, 923, **8**, 591.
25. Stadie, W. C.: A method for the determination of methemoglobin in blood. *J. Biol. Chem.*, 1920, **41**, 237.
26. Stoddard, J. L., and Adair G. S.: The refractometric determination of hemoglobin. *J. Biol. Chem.*, 1923, **57**, 437.
27. Terrill, E. H.: On the colorimetric determination of hemoglobin with especial reference to the production of stable standards. *J. Biol. Chem.*, 1922, **53**, 179.
28. Van Slyke, D. D., and Hiller, A.: Gasometric determination of hemoglobin by the carbon monoxide capacity method. *J. Biol. Chem.*, 1928, **78**, 807.
Gasometric control of standards for the Palmer hemoglobin method. *J. Biol. Chem.*, 1929, **84**, 211.

29. Van Slyke, D. D., and Stadie, W. C.: The determination of the gases in the blood. *J. Biol. Chem.*, 1921, **49**, 1.
30. Williamson, C. S.: Influence of age and sex on hemoglobin. A spectrophotometric analysis of nine hundred and nineteen cases. *Arch. Int. Med.*, 1916, **18**, 505.
31. Wolter, O.: Über das Harneisen. I. Die Bestimmung des Eisens im Harm. *Biochem. Z.*, 1910, **24**, 108.
32. Wong, S. Y.: Colorimetric determination of iron and hemoglobin in blood. II. *J. Biol. Chem.*, 1928, **77**, 409.
33. Wu, H.: Studies on hemoglobin. I. The advantage of alkaline solutions for colorimetric methods for the determination of hemoglobin. *J. Biochem.* (Japan), 1922, **2**, 173.
34. Wu, H.: III. An ultra-micro-method for the determination of hemoglobin as a peroxidase. *J. Biochem.*, (Japan), 1922, **2**, 189.

CHAPTER XXI

Proteins of Urine, Blood Plasma, and Body Fluids

DISCUSSION

Determination of total proteins

The methods proposed for determination of urine and plasma proteins have been partly chemical and partly physical.

Chemically the proteins have been determined by coagulating them and weighing the coagulum, or determining its nitrogen content by the Kjeldahl method, or by redissolving it and estimating the protein content by a quantitative application of one of the protein color reactions. These procedures, especially the gravimetric and Kjeldahl, are capable of yielding uniform results.

Because of the striking physical peculiarities of the plasma proteins and the fact that they constitute the greater part of the plasma solids, the problem of finding conditions under which one of the easily determined physical constants of the plasma could be used as a measure of the protein content has been an inviting one. Starlinger and Hartl (26) have reviewed methods based on interferometric, polarimetric, viscosimetric and refractometric measurements, all of which are influenced chiefly by the proteins among the plasma constituents. Of these measurements only the refractometric has proved sufficiently practicable to win any general use. It was introduced by Reiss (22) and was developed by Robertson (23) into a procedure for determining not only the total proteins, but also the albumin and globulin fractions separately. Neuhausen and Rioch (21) found that when proper constants were used in the calculations refractometric and chemical methods in normal plasma agreed fairly well. Linder, Lundsgaard and Van Slyke (19), however, found refractometric deviations from Kjeldahl results as great as 1.5 grams of total protein per 100 cc. even in normal plasma. Guillamin, Wahl and Laurencin (11) have obtained by the refractometric method similar errors in normal plasma, and in the plasma of edematous patients have found by refractometer up to double the amount of protein actually present by chemical analysis. This type of pathological plasma is excessively rich in lipoids and it is presumably by their effect on the refractive index that the gross errors arise. It is in precisely this type of plasma that the protein contents are of especial clinical interest. We shall accordingly not describe the refractometric methods.

Of the physical characteristics the specific gravity has proved to parallel the protein content closely enough so that specific gravity determinations are of practical value in clinical studies. Moore and Van Slyke (20) in a study of plasma changes in nephritis have found that specific gravity changes closely parallel protein changes, and that both show a high degree of correlation with the tendency to formation of non-cardiac edema. We shall accordingly describe the simple specific gravity method used by them.

Separation of protein fractions

Methods for determination of the separate proteins or protein fractions depend upon their separation by fractional precipitation followed by chemical determination, gravimetric, Kjeldahl, or colorimetric. Fibrinogen is the most readily precipitated of the proteins. It coagulates with heat at the lowest temperature, is salted out by the lowest concentrations of electrolytes, and from oxalate plasma coagulates spontaneously when the oxalate is precipitated with calcium. All of these procedures have been used to separate fibrinogen from the other proteins (see Foster and Whipple (8)).

Both fibrinogen and the other globulins are precipitated by half saturation with ammonium sulfate, by saturation with magnesium sulfate, or by the presence of 22 grams of anhydrous sodium sulfate per 100 cc.

In the filtrate from the globulins, according to present methods, all the protein is determined as albumin.

Only in the case of fibrinogen can the separation be considered a sharp one. The quantitative separation of the other globulins from albumin would require repeated precipitation and resolution to remove adsorbed albumin from the precipitate, and would involve corrections for the measurable solubility of the globulins in the precipitating salt solutions. By convention filtrate protein from a single precipitation with half saturated $(NH_4)_2SO_4$, saturated $MgSO_4$, or 22 per cent Na_2SO_4, is calculated as "albumin." The separation is approximate enough to yield at least fractions which have quite different clinical significance in both urine and blood plasma.

Hammarsten (12), in 1878, first showed that globulins could be salted out from other proteins of blood serum. The conditions under which such separation can be best effected have, since then, been carefully defined by many investigators, and expecially by Howe (15, 16, 17) and Starlinger and Hartl (26). The use of magnesium sulfate entails the addition to serum of both a saturated solution of the salt and additional solid salt. Ammonium sulfate makes subsequent determination of the proteins by Kjeldahl difficult, but can be employed to advantage if the proteins are to be determined by some other method with which the presence of ammonia does not interfere. Howe (15), in 1921,

proposed sodium sulfate. This salt is less soluble than ammonium sulfate and at room temperature can not be made sufficiently concentrated to precipitate globulin completely. At incubator temperature, however, its solubility is sufficiently increased to permit its use as a globulin precipitant. Howe has also defined the concentrations of this salt that are required for the precipitation of fibrinogen and the individual globulin fractions. These are given in table 59. Since the urine proteins have the same properties as the plasma proteins, the figures presumably hold for both.

Albuminous urines usually contain several times as much albumin as globulin, and apparently no chemically determinable amounts of fibrinogen. Usually only total urine proteins are determined: rarely the albumin and globulins are estimated separately for clinical purposes.

In plasma this separation is much more frequently used, and separate determinations of fibrin are not uncommon.

TABLE 59

CONCENTRATIONS OF SODIUM SULFATE REQUIRED TO PRECIPITATE THE DIFFERENT PROTEINS OF BLOOD PLASMA

	Na_2SO_4 CONCENTRATION	
	molar	*per cent*
Fibrinogen........	0.76	10.6
Euglobulin........	1.00	14.2
Pseudoglobulin I..	1.25	17.7
Pseudoglobulin II.	1.50	21.5

Choice of methods

For accuracy the gravimetric methods stand first. In the Kjeldahl procedures, the errors of the Kjeldahl determination are added to those of isolation, but since even micro Kjeldahl analyses can be done with errors not exceeding 1 per cent, protein estimations by this method are but slightly less accurate than the gravimetric.

The colorimetric methods are, in the authors' experience, liable to errors up to 10 per cent, likewise the rapid sedimentation test which is described for urine proteins.

For *urine total proteins,* if accurate results are desired one should therefore use either the gravimetric or Kjeldahl method. If approximate results suffice, the sedimentation method is as exact as the colorimetric, and much more simple, rapid, and convenient.

For *urine albumin and globulin* determined separately only colorimetric methods are available. Results of clinical significance connected with gross

changes of the albumin-globulin ratio have been obtained with these methods. To the uncertainty of the colorimetric technique, however, is added that due to partial solubility of the globulin under greatly varying conditions of dilution. The writers therefore present these methods without attempting even an approximate estimate of their accuracy. The procedures provide means for comparing results with others by the same methods in the literature, and are described merely for that purpose.

For *plasma proteins* the same remarks hold with regard to the relative accuracy of gravimetric, Kjeldahl, and colorimetric methods. The separation of aluminum and globulin in plasma, however, appears to be a somewhat more exact procedure than in urine, because in plasma the concentrations of the proteins are greater and less variable than in urine. For total protein and fibrin in plasma the gravimetric and Kjeldahl procedures are accurate. The specific gravity method is the best rapid indirect procedure that the writers have encountered for indicating the approximate total protein content.

TOTAL PROTEINS OF URINE. GRAVIMETRIC METHOD OF FOLIN AND DENIS (6)

Reagents

Five per cent acetic acid. Five cubic centimeters of glacial acetic acid diluted with water to 100 cc.

0.5 per cent acetic acid. Five cubic centimeters of glacial acetic acid diluted to 1 liter.

Fifty per cent ethyl alcohol.

Procedure

Ten cubic centimeters of urine are pipetted into an ordinary conical centrifuge tube that has been previously weighed. To this urine is then added 1 cc. of 5 per cent acetic acid, and the tube is allowed to stand fifteen minutes in a beaker of boiling water to coagulate the proteins. The tube is then centrifuged for a few minutes. The supernatant liquid is poured off and the precipitate is stirred up with about 10 cc. of hot 0.5 per cent acetic acid and again centrifuged. The supernatant liquid is again poured off, and the precipitate in the tube again washed, this time with 50 per cent alcohol. After centrifuging and pouring off the supernatant liquid for a third time the tube is placed for 2 hours in an air bath at 100° to 110°, then cooled in a desiccator and weighed.

TOTAL PROTEINS OF URINE. MICRO KJELDAHL METHOD

The proteins are precipitated with the Folin-Wu tungstic acid precipitant as used by Wu and Ling (30) in the first step of their colorimetric determination. The proteins are purified free from other nitrogenous substances by redissolving the precipitate in sodium tungstate solution, and reprecipitating by adding sulfuric acid. The nitrogen in the precipitate is determined by micro Kjeldahl analysis.

Reagents

Ten per cent sodium tungstate and two-thirds normal sulfuric acid used in Folin-Wu blood protein precipitation (see p. 65).

Sodium hydroxide. 5 N solution.

Procedure

Transfer to a graduated 15 cc. centrifuge tube a volume of filtered urine estimated to contain 2 to 10 mg. of protein. For a very heavy proteinuria (1 to 2 grams per 100 cc.) the sample required is 0.5 cc., for a lighter proteinuria 1 to 5 cc. Dilute with water to 10 cc. Add 2 cc. of 10 per cent sodium tungstate and 2 cc. of $\frac{2}{3}$ N sulfuric acid. Stir the mixture with a slender glass rod and centrifuge. In case the supernatant fluid is milky in appearance, which occurs only with alkaline urines, add a few more drops of $\frac{2}{3}$ N sulfuric acid, stir, and centrifuge again. Carefully decant the clear supernatant fluid. Add 1 cc. of sodium tungstate solution to the precipitate in the tube and stir until it has dissolved. Dilute to 10 cc., add 1 cc. of $\frac{2}{3}$ N sulfuric acid, stir, centrifugate, and decant again. Repeat the procedure once more to remove completely non-protein nitrogenous substances. If the volume of the centrifuged precipitate exceeds 1 cc. dissolve the precipitate in a few cubic centimeters of water plus a few drops of strong NaOH, dilute to 10 cc., and take for Kjeldahl analysis an aliquot part that would give about 0.5 cc. of protein tungstate precipitate.

For analysis either the entire precipitate or an aliquot of its solution, taken as above described, is transferred to a tube or micro Kjeldahl flask, and the nitrogen content is determined as described for micro Kjeldahl analyses of protein-containing material in the chapter on nitrogen.

TOTAL PROTEINS OF URINE, RAPID APPROXIMATE SEDIMENTATION METHOD OF SHEVKY AND STAFFORD (24)

Esbach's method of estimating protein content by the volume of picric acid protein precipitate, despite the known fact that it may give results

varying from half to twice the true amounts, has held its own through years because of its convenience. Finally Tsuchiya (28) showed that an alcoholic solution of phosphotungstic and hydrochloric acids used in the same way gave much more accurate results, presumably because of the greater specific gravity of the phosphotungstate precipitate. Shevky and Stafford (24) in Addis' laboratory further increased the accuracy by throwing down the precipitate in graduated centrifuge tubes instead of depending upon sedimentation by gravity. Even by this method the error may exceed 10 per cent; but it is extremely rapid and simple, and about as exact as the colorimetric procedures.

Reagents

Tsuchiya's solution. Fifteen grams of phosphotungstic acid, 50 cc. of concentrated hydrochloric acid, and 1 liter of 95 per cent alcohol are mixed.

Procedure

The following procedure, used in routine work by E. M. MacKay (personal communication) deviates in some details from the original description. The first step is dilution of the urine. Nephritic urines are usually diluted 10-fold. In urines with very scanty protein content less dilution or none at all will give more exact results. Occasionally a urine is encountered with more than 2.8 per cent of protein, which is the maximum which can be determined with a 10-fold dilution. In such a case a fresh sample is diluted 20-fold and the determination repeated.

Of the diluted urine 4 cc. are measured into the special graduated centrifuge tube described below, the 4-cc. mark on the tube itself serving for the measurement. Then Tsuchiya's reagent is added until the tube is filled to the 6.5-cc. mark. The tube is closed and the contents are mixed by inverting slowly 3 times. After the mixing the tube is allowed to stand exactly 10 minutes, when it is centrifuged for exactly 10 minutes at 1800 revolutions per minute. The volume of precipitate is read on the scale.

The calculation follows the formula:

$$\text{Grams of protein per liter urine} = \text{cubic centimeters of precipitate} \times 7.2 \times \text{dilution}$$

where "dilution" indicates the number of times the urine was diluted before the sample was measured into the tube.

The centrifuge tube, made of Pyrex glass, is shown in figure 81. It was designed by Dr. E. M. MacKay. It is graduated into 0.01 cc. divisions

at the bottom for the first 0.4 cc. From there to 1.0 cc. it is graduated to 0.1 cc. divisions. Above the 1.0-cc. mark it is graduated at 2.0, 3.0, 4.0 and 6.5 cc. The inner diameter of the wide upper part of the tube is 15 mm. mm., that of the narrow lower part divided into 0.01 cc. divisions is 3.8 mm. All of the calibration marks are rings which go completely around the tube.

TOTAL PROTEINS, GLOBULINS, AND ALBUMIN IN URINE. HILLER'S (13, 14) MODIFICATION OF AUTENRIETH'S (2) COLORIMETRIC METHOD

Albumin and globulin are separated by precipitating the latter with sodium sulfate, as in Howe's technique for plasma protein separation. The

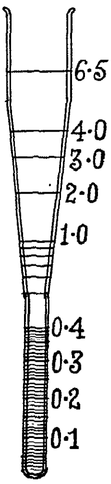


FIG. 81. Centrifuge tube for the determination of protein in urine by the method of Shevky and Stafford.

separated proteins are determined by the colorimetric method of Autenrieth (1, 2), which depends on the development of the biuret color by proteins treated with coper sulfate and alkali.

The chief disadvantages of the original Autenrieth method were the lack of satisfactory standards and the tedious technique of precipitating and washing the proteins. In Hiller's method the standards are prepared from solutions of pure biuret. One milligram of biuret gives a color equal to that produced by 0.924 mg. of either total urinary protein or the albumin in the sodium sulfate filtrate from the globulins. Instead of heat and acetic acid trichloroacetic acid is used to precipitate total protein and albumin, which are centrifuged instead of being washed on a filter.

Reagents

Ten per cent trichloroacetic acid solution.

Three per cent sodium hydroxide solution.

Thirty per cent sodium hydroxide solution.

Twenty per cent copper sulfate solution, containing 20 grams $CuSO_4 \cdot 5H_2O$ per 100 cc. of solution.

Forty-four per cent sodium sulfate solution containing 44 grams anhydrous Na_2SO_4 per 100 cc. of solution. This solution is saturated at 37° and must be kept at that temperature to prevent crystallization. The sodium sulfate solution must be neutral to litmus.

Standard biuret solution. Dissolve 0.4000 grams of biuret in distilled water and dilute to a volume of 150 cc. This solution will keep in the ice box at least a month.[1]

Procedure

Preparation of urine samples. Adjust a portion of urine (50 to 200 cc.) to a pH of about 7.4, slightly alkaline to sensitive litmus paper. The reaction may be adjusted with more certainty by removing drops and testing with phenol red. Filter if not perfectly clear. This same specimen can now be used for the precipitation of globulin and for total protein.

Determination of total protein

Measure 2 cc. of the specimen into a graduated centrifuge tube, add an equal volume of 10 per cent trichloroacetic acid, mix with a narrow glass rod, and centrifuge five minutes. If the volume of precipitate is between 0.2 and 0.6 cc., the amount of protein in it can be read against the standard described below and the analysis is continued as described in the next paragraph. If the volume of precipitate is larger or smaller a second precipitation is performed, with enough urine to yield a precipitate of between 0.2 and 0.6 cc.

Pour off the supernatant fluid, draining as dry as possible. Dissolve the precipitate in about 3 cc. of 3 per cent sodium hydroxide solution and wash into a 10-cc. graduated cylinder with portions of 3 per cent sodium hydroxide until the volume has reached about 9 cc. Add 0.25 cc. of 20 per cent copper sulfate solution, dilute to 10 cc. with 3 per cent sodium hydroxide. Mix thoroughly by shaking, let stand 10 minutes, centrifuge, and compare the supernatant fluid in a colorimeter against a standard prepared at the same time.

[1] Kahlbaum's biuret is satisfactory.

To prepare the standard color solution measure 5 cc. of the standard biuret solution, containing 13.33 mg. of biuret, equivalent to 12.3 mg. of protein, into a 10 cc. graduated cylinder. Add distilled water to 8 cc., add 1 cc. of 30 per cent sodium hydroxide, 0.25 cc. of 20 per cent copper sulfate solution, then dilute to 10 cc. with water. Mix thoroughly, let stand 10 minutes and centrifuge. Transfer the supernatant fluid to the colorimeter cup, and compare with the solution of urine protein, setting the depth of the standard column at 15 mm.

Calculation of total protein

$$\frac{12.3\ S}{U\ V} = \text{grams of protein per liter of urine.}$$

U is the reading of the unknown solution, S the reading of the standard, and V the cc. of urine used.

Precipitation of globulin

To 10 cc. of urine prepared as described above, add 10 cc. of 44 per cent sodium sulfate solution, mix well and place in an incubator at 37°C. for three hours. Filter until a perfectly clear filtrate is obtained.

Determination of albumin

With the filtrate from the sodium sulfate precipitation proceed as described under "determination of total protein," performing the precipitation tentatively with a volume of filtrate 4-fold that of the urine taken for total protein determination.

Calculation of albumin

$$\frac{12.3\ S}{U\ V} = \text{grams of albumin per liter of urine.}$$

Calculation of globulin

The globulin is estimated by difference.

$$(\text{Total protein}) - (\text{Albumin}) = (\text{Globulin}).$$

BENCE-JONES PROTEINS IN URINE. GRAVIMETRIC METHOD OF FOLIN AND DENIS (7)

The presence of such proteins can be detected qualitatively by the characteristic temperature reactions. If the urine containing the protein is gradually heated clouding begins at from 40° to 55°C., increasing in

intensity, until at about 65°–70°C. precipitation becomes complete and the coagulated protein settles out sharply. As the temperature continues to rise the precipitate begins to redissolve between 95° and 100°C. and dissolves completely after the solution has boiled for a short time. When it is recooled the precipitate reappears slightly below 100°C., and then redissolves when the temperature has fallen to somewhere between 40° and 55°. These reactions, especially the clearing on boiling, are sharply demonstrable only if the urine is distinctly acid; the tests are therefore best carried out after the urine has been acidified with acetic acid. If the concentration of protein is great, the coagulum formed at low temperatures may dissolve with difficulty or incompletely on boiling. In this case the reactions become clearer if the urine is diluted with other, protein-free urine or acidulated salt solution before the tests are made. If the urine contains other proteins these mask the characteristic reaction by coagulating when the urine is boiled. In this case these proteins must be precipitated by heat and removed by filtration, and the tests for Bence-Jones protein must be applied to the serum-protein-free filtrate. As an alternative procedure the Bence-Jones protein may be coagulated from slightly acid solution at a temperature of 60° to 65°, removed by filtration, and resuspended in protein free urine or acidulated salt solution.

Reagents

A 5 per cent solution of acetic acid.
A 50 volume per cent solution of alcohol.

Procedure of Folin and Denis

To 10 cc. of urine in a weighed centrifuge tube add 1 cc. of 5 per cent acetic acid. Let the tube stand overnight in a water-bath at a temperature of 60°. The next morning centrifuge the mixture and decant the supernatant liquid. To the precipitate in the tube add 10 cc. of 50 per cent alcohol. Stir the sediment up well, recentrifuge and again decant the supernatant liquid. Repeat the washing with alcohol once. After decanting the alcohol dry the precipitate in an oven at a temperature of 100° to 110°. Weigh the tube and precipitate.

One hundred times the weight of the precipitate is the amount of the protein in 1 liter of urine.

Taylor and Miller (27) prefer precipitation from cold acid solution by half-saturation with sodium sulfate. The precipitate, isolated by filtration on a hardened filter, is washed in succession with 95 per cent alcohol, absolute alcohol, alcohol and ether, and ether. It is finally dried and weighed.

TOTAL PROTEINS OF SERUM OR PLASMA. GRAVIMETRIC METHOD OF BIERRY AND VIVARRO (3) MODIFIED BY GUILLAUMIN, WAHL, AND LAURENCIN (11)

Reagents

Acetone.
0.1 N acetic acid.
0.6 per cent sodium chloride.

Procedure

Two or 3 cc. of serum or plasma are pipetted into 10 cc. of acetone in a centrifuge tube. The acetone precipitates the proteins and dissolves the lipoids. The mixture is stirred with a rod and permitted to stand three hours. It is then centrifuged, the supernatant fluid is decanted, and the precipitate is washed twice by stirring it up with fresh acetone and centrifugating. A desirable precaution is to pour the washings through a small filter so that any suspended particles of precipitate may be retained.

With the same stirring rod the precipitate is mixed with 20 cc. of 0.6 per cent sodium chloride solution, whereby partial solution is obtained. The solution and undissolved proteins are washed into a porcelain dish, a few drops of methyl red are added and then acetic acid until the pH is reduced to approximately 4.7. The dish is covered and heated an hour on a boiling water bath to coagulate the proteins.

The protein precipitate is washed into a weighed Gooch crucible with hot water followed by alcohol and ether, and is dried over night in an oven at 105°. After cooling in a desiccator it is weighed.

This is the most rigidly exact of plasma protein methods, because the acetone treatment removes lipoids, and the acetic acid coagulation removes salts, from the protein precipitate. In the other methods, described in the following pages, the lipoids are precipitated with the proteins, and their nitrogen content probably causes a slight plus error in the protein figure.

TOTAL PLASMA OR SERUM PROTEINS. KJELDAHL METHOD

The nitrogen is determined by macro Kjeldahl analyses in 1.0 cc. of serum or plasma, or by micro Kjeldahl in 0.1 cc. The non-protein nitrogen is also determined, as described in the nitrogen chapter.

$$\text{Total nitrogen} - \text{NPN} = \text{protein nitrogen.}$$

$$\text{Grams of protein nitrogen} \times 6.25 = \text{grams of protein.}$$

In ordinary serum or plasma the protein nitrogen is about 1000 mg. per 100 cc. and the non-protein (or rather non-colloid) nitrogen only 35 ± 10 mg. or 3.5 ± 1.0 per cent as much. Therefore, when there is no nitrogen retention, one can, with an error not exceeding 1 per cent, omit the non-protein nitrogen determination, and assume it to be 35 mg. per 100 cc. for the above protein nitrogen determination.

TOTAL PROTEIN CONTENT OF PLASMA ESTIMATED FROM SPECIFIC GRAVITY. MOORE AND VAN SLYKE (20)

Procedure

Blood is drawn into a vessel containing about 1 mg. of heparin per cubic centimeter of blood. The blood is centrifuged, preferably in a stoppered tube to prevent evaporation. For determining the specific

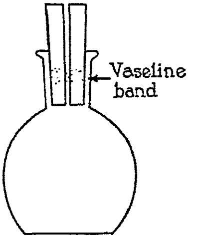


FIG. 82. Flask for the determination of the specific gravity of plasma by the method of Moore and Van Slyke (20).

gravity, bottles of the common type shown in figure 82 and of approximately 2 cc. capacity are used.[2] When such a bottle is filled with solution and closed with a dry glass stopper slight amounts of fluid are drawn up by capillary attraction between the stopper and the neck of the flask, and evaporate when they reach the groove at the top of the neck. When the total amount of fluid is only 2 cc. the loss by this evaporation is sufficient to cause significant errors. It may be prevented, however, by placing a thin ring of vaseline about 3 mm. wide around the stopper as shown in figure 82. Such a band of vaseline adds 0.1 to 0.5 mg. to the weight of the bottle. For each determination the bottle with the vaselined stopper is first weighed empty. It is then filled with the plasma at approximately room temperature. A small drop of plasma pressed out of the capillary through the stopper is left

[2] These special bottles can be obtained from Eimer and Amend in New York.

on the latter for 10 or 15 minutes, during which the bottle stands in the balance case to come to its temperature. After this interval the drop is wiped off cleanly, the bottle is wiped with a clean cloth, and is weighed to within 0.1 mg. The specific gravity is calculated as

$$\text{Specific gravity} = \frac{\text{weight of plasma held by bottle at } 20^\circ}{\text{weight of water held by bottle at } 20^\circ}$$

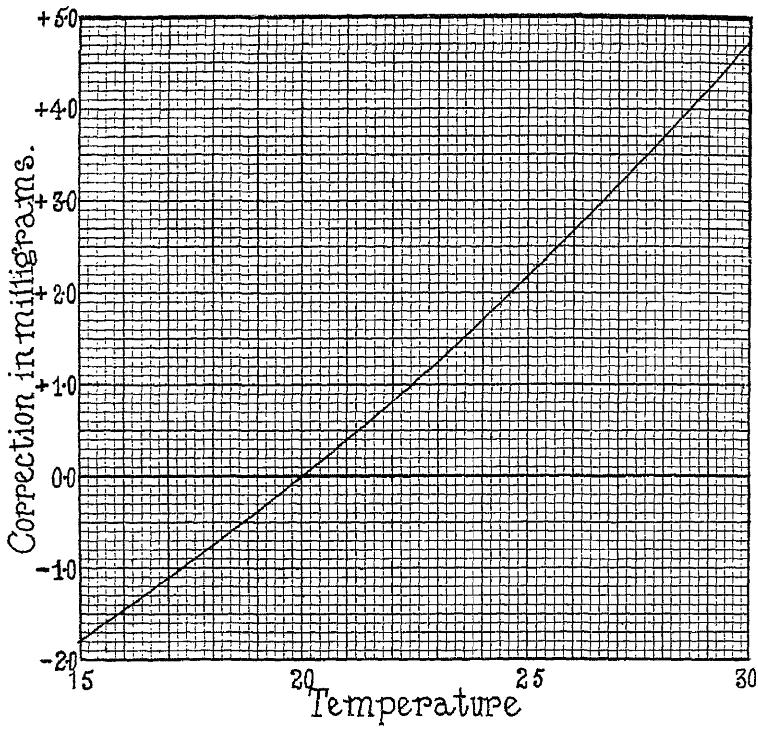


FIG. 83. Chart of temperature correction factors for the specific gravity of plasma as determined by the method of Moore and Van Slyke. The curve indicates the number of milligrams that are to be added to, or subtracted from, the determined weight of the plasma in a bottle of 1.9 to 2.1 cc. capacity to give the weight of plasma at 20°C.

It is convenient to weigh the filled bottles at room temperature as above described, and estimate the weight at 20° by adding or subtracting a correction in milligrams, rather than to bring to 20° for each weighing.

The corrections for a bottle of 1.9 to 2.1 cc. capacity are given by the curve in figure 83. For bottles of greater or less capacity the correction taken from figure 83 is multiplied by the factor

capacity of bottle in cubic centimeters

The protein content with a maximum error of 0.6 gram per 100 cc. can be estimated from the specific gravity, G, by the formula,

$$\text{Grams of total protein per 100 cc. of plasma} = 343\ (G - 1.007).$$

TOTAL PROTEIN, GLOBULIN, AND ALBUMIN IN SERUM OR PLASMA, HOWE'S KJELDAHL METHOD (16)

Reagents

0.9 per cent solution of sodium chloride.

Twenty two per cent solution of sodium sulfate. 22 gm. anhydrous Na_2SO_4 to 100 cc. This solution is supersaturated at room temperature and must, therefore, be kept well-stoppered, and at 38°.

Procedure

The ***plasma non-protein nitrogen*** is determined by the usual micro Kjeldahl technique (see non-protein nitrogen determination, p. 536) in a tungstic acid or trichloroacetic acid filtrate.

Total plasma nitrogen. For ***macro determination*** dilute 1 cc. of plasma to 25 cc. with 0.9 per cent NaCl solution and analyze 10 cc. aliquots by the usual macro Kjeldahl technique. For the digestion the Arnold-Gunning mixture plus peroxide or persulfate, as described in the nitrogen chapter, is used. The titrations are made with 0.05 N alkali and acid. A blank determination is run on all the reagents, including the sodium chloride.

For ***micro determination*** 0.5 cc. of plasma is diluted to 5 cc. and portions of 1 cc. are used for micro Kjeldahl determinations.

Plasma albumin plus non-protein nitrogen. Plasma, pipettes, flasks, and funnels must all be kept constantly at 37° to 38° in order to prevent crystallization of the sodium sulfate from the solution.

For ***macro determination.*** One cubic centimeter of plasma is placed in a small Erlenmeyer flask, and 30 cc. of the 22 per cent sodium sulfate solution are added, and a small crystal of thymol. Mix, stopper the flask, and allow it to stand in an incubator at 38° for three hours or

longer. Then filter in the incubator through a hardened filter paper. The filtrate contains the albumin and non-protein nitrogen. Certain plasmas do not filter clear at once. In these cases it is necessary to pass the cloudy filtrate through the filter two or more times until a clear filtrate is obtained. Occasionally from highly lipemic plasmas a clear filtrate can not be obtained. In such a case one must analyze the still somewhat cloudy filtrate. The suspended matter appears to be fat which does not significantly affect the nitrogen figures. Of the filtrate 10 cc. portions are analyzed by the macro Kjeldahl procedure. 0.02 N acid and alkali are used for the titration. A blank determination must be run which includes all the reagents, including the sodium sulfate.

For ***micro determination*** 0.5 cc. of plasma is mixed with 15 cc. of sodium sulfate and the rest of the procedure is carried through in the same manner. Five cubic centimeters aliquots are used for micro Kjeldahl analyses.

Calculation

The nitrogen values are calculated in the usual manner as described in the nitrogen or gasometric chapter.

Grams nitrogen per 100 cc. of plasma are calculated by multiplying the grams of nitrogen found in the sample analyzed by $100/V$, where V indicates the cubic centimeters of plasma represented in the sample. In the determination of the total nitrogen the value of $100/V$ is 250 for the macro Kjeldhal, 1000 for the micro. In the analyses of the albumin + NPN in the filtrate the value of $100/V$ is 310 for the macro Kjeldahl, 620 for the micro.

The different protein fractions are calculated as follows:

$$\text{Albumin N} = (\text{Albumin N} + \text{NPN}) - (\text{NPN})$$

$$\text{Globulin N} = (\text{Total N}) - (\text{Albumin N} + \text{NPN})$$

$$\text{Total protein N} = (\text{Total N}) - (\text{NPN})$$

Protein is calculated as nitrogen $\times$ 6.25. "NPN" indicates non-protein nitrogen.

Remarks

In the laboratory of one of the authors the macro modification here described of Howe's technique is used. In the laboratory of the other author the micro modification, with micro Kjeldahls by the gasometric method

described on page 354 is employed. Both procedures have proved satisfactory in some years of routine.

The point in the analysis that is most likely to cause variation in the results is the precipitation of the globulins with sodium sulfate. It is an empirical approximate separation rather than a sharply quantitative one, and deviations in results between two duplicate filtrates are more likely to occur than differences between Kjeldahl analyses of the same filtrate. It is therefore essential to carry out two duplicate precipitations of the globulins. One analysis of the filtrate of each is made. If they do not agree the Kjeldahls are repeated, to find whether the difference is due to these or to the filtrations.

TOTAL PROTEIN, GLOBULIN, AND ALBUMIN IN SERUM OR PLASMA. COLORIMETRIC METHOD OF WU AND LING (29, 30) AS MODIFIED BY GREENBERG (10)[3]

Wu (29) introduced the procedure of determining plasma proteins colorimetrically by means of the phenol molybdate-tungstate reagent, which presumably reacts with the phenol group of the tyrosine in each protein. Wu and Ling (30) improved the method, and Greenberg (10) has further modified it by applying the new improved phenol reagent of Folin and Ciocalteu (5) and using the sodium sulfate procedure of Howe (15) for separating proteins. Greenberg estimates the accuracy of the method to be 5 per cent of the amounts determined.

Reagents

Sodium sulfate solution. 22.5 grams anhydrous Na_2SO_4 per 100 cc. This solution must be kept at 38° to prevent crystallization.

Sodium hydroxide. 5 N solution.

Tyrosine standard. 200 mg. tyrosine made up to 1 liter in 0.1 N hydrochloric acid.

Folin's colorimetric phenol reagent. The Folin and Ciocalteu (5) improvement of this reagent described in the chapter on phenol methods, p. 655.

Procedure

Pipette 0.5 cc. of plasma from a calibrated pipette into a 15 or 20-cc. test tube. Add exactly 9.5 cc. of 22.5 per cent sodium sulfate solution

[3] Note added to proof. The writers have been advised by a colleague that this colorimetric method, when applied to the plasma of patients with nephrosis, gives incorrect albumin:globulin ratios. Apparently the chromogenic power of the proteins, in either the albumin or the globulin group, or both, can suffer marked alteration in disease.

with a pipette of that volume or from a burette. Stopper, agitate thoroughly and set aside for about two hours in an incubator at 37° to 38° to allow coagulation of the globulin. At the end of this period, filter into another test tube, using a fairly retentive filter paper (Whatman No. 42 is satisfactory). Examine to see that the filtrate is clear; if not, pour back on to the filter paper. After the filtering is nearly complete, remove the tube containing the filtrate to be used for albumin analysis. The residue of globulin in the test tube in which the precipitation was carried out is now washed on to the filter paper by two washings with 3 cc. each of sodium sulfate solution.

The globulin precipitate is then washed twice more with 3 cc. portions of sodium sulfate solution. The two protein fractions are now estimated as follows.

Globulin. The funnel with filter paper containing the globulin precipitate is placed over a 50-cc. volumetric flask, a small hole is punched in the bottom of the filter paper with a wire or drawn out glass rod, and the globulin is dissolved and washed into the volumetric flask by a stream of approximately 0.01 N sodium hydroxide from a wash bottle. The washing is completed with distilled water until the flask is about half full. The filter paper is then unfolded and examined to see that all the globulin is dissolved. To the flask there are now added 2 cc. of 5 N NaOH and 3 cc. of the phenol reagent. The flask must be agitated while the phenol reagent is being added, to prevent a large local excess, which may result in a turbid precipitate. The flask is now filled to the mark with distilled water and the contents are thoroughly mixed. A standard is prepared at the same time by pipetting 4 cc. of the standard tyrosine solution into another 50 cc. volumetric flask, adding about 25 cc. of water, then 2 cc. of sodium hydroxide, and 3 cc. of phenol reagent, agitating the contents of the flask during the course of adding the reagents, and finally filling with water to the graduation mark. After both have stood for from five to ten minutes to insure full color development the solutions are placed in colorimeter cups, the standard is set at the 20 mm. mark on the colorimeter, and the unknown compared with it.

Albumin. For the determination of the albumin, a 5-cc. aliquot of the filtrate is pipetted into a 50-cc. volumetric flask, about 25 cc. of water are added, then 2 cc. of 5 N sodium hydroxide and 3 cc. of the the phenol reagent. The flask is then filled with distilled water to volume and the contents are thoroughly mixed. A standard is prepared at the same time with 4 cc. of standard tyrosine solution. After

five to ten minutes the colors are read with the standard set at the 20-mm. mark on the colorimeter.

Total plasma or serum protein. Determination of total plasma protein can be carried out according to the procedure given above, with either 0.2 cc. of plasma measured directly in a 0.2 cc. pipette or, perhaps more accurately, as a 2 cc. aliquot of 1 cc. of plasma that has been diluted in a 10 cc. volumetric flask. The comparison is carried out in the usual manner against a 4 cc. portion of standard tyrosine solution. This determination of total protein gives the possibility of determining the globulin, not directly, but by subtracting the value of the albumin fraction from the total protein. It is more accurate, however, to determine both albumin and globulin independently as described above.

Calculations

The general formula for calculating the percentage of protein in plasma or serum from the colorimetric reading is

$$\frac{S}{U} \times T \times \frac{100}{V} \times \frac{f}{1000} = \text{grams of protein per 100 cc. of plasma.}$$

In this formula S is the point at which the standard is set, U the reading of the unknown, T the milligrams of tyrosine in the standard solution, V the volume of plasma in cubic centimeters represented in the sample used, and f the number of milligrams of the protein which give the same amount of color as 1 mg. of tyrosine. The standard in each of these analyses contains 0.8 mg. of tyrosine, hence $T = 0.8$. The values of f are: total protein, 16.0; albumin, 16.8; globulin, 14.4. The volumes V of plasma represented in the samples analyzed are: total protein, 0.2 cc.; albumin, 0.25 cc.; globulin, 0.5 cc. With the above numerical values inserted the formula becomes, for the respective protein determinations:

$$2.30 \times \frac{S}{U} = \text{grams of globulin per 100 cc.}$$

$$5.38 \times \frac{S}{U} = \text{grams of albumin per 100 cc.}$$

$$6.4 \times \frac{S}{U} = \text{grams of total protein per 100 cc.}$$

Precautions

The color obtained with the phenol reagent is dependent on the age of the plasma sample. The amount of color obtained decreases with time as the plasma stands. On this account freshly obtained plasmas that have stood no longer than over night should be used for the analysis. The alkali and phenol reagent are to be in the proportions recommended. These amounts were found to give optimum color development under the conditions of the analytical method.

FIBRIN OF PLASMA

For the separation of fibrin from plasma most of the recent methods utilize the procedure developed by Cullen and Van Slyke (4) in which calcium chloride is added to oxalated plasma in amounts to supply the optimum excess of dissolved calcium for formation of a fibrin clot. Thirty-fold dilution of the plasma with 0.8 per cent NaCl solution was found to give an optimum dilution. Gram (9) has developed a similar procedure for use with citrated blood.

Precipitation of fibrin. Cullen and Van Slyke (4)

Reagents

0.8 per cent NaCl solution.

Calcium chloride solution containing 2.5 grams of anhydrous $CaCl_2$ or 5 grams of crystalline $CaCl_2 \cdot 6H_2O$ per 100 cc.

Procedure

The amount of potassium oxalate added to the whole blood should be approximately 5 mg. per cubic centimeter. Of the centrifuged plasma 5 cc. is taken for analysis if the fibrin is to be determined by macro Kjeldahl analysis, 2 cc. if it is to be weighed, and 1 cc. if it is to be determined by micro Kjeldahl or Wu's colorimetric procedure.

For each cubic centimeter of plasma 30 cc. of 0.8 per cent NaCl solution are added, then 1 cc. of the calcium chloride solution. The solutions are mixed and allowed to stand for about an hour for coagulation to become complete. The clot is then treated as follows, according to the method by which it is to be determined.

Macro Kjeldahl determination of fibrin in precipitate. Cullen and Van Slyke (4)

The clot from 5 cc. of plasma is transferred to a filter paper in a funnel and is washed 5 times with 0.8 per cent NaCl solution. At

each washing sufficient solution is poured on to the filter to cover the clot and is allowed to remain there for 10 minutes by closing the outlet of the funnel with a piece of rubber tubing and a pinch-cock. The filter paper and the washed fibrin are transferred to a Kjeldahl flask and analyzed by an appropriate Kjeldahl technique. The fibrin is calculated as nitrogen times 6.25.

Gravimetric determination of fibrin precipitate. Foster and Whipple (8)

The clot obtained by Cullen and Van Slyke's method from 2 cc. of plasma is freed as completely as possible from fluid by gentle manipulation and pressure with a glass rod and is then washed in distilled water. The clear fibrin is then placed in a small porcelain crucible and dried at 110° for ten hours. The crucible is cooled in a desiccator and weighed. It is then ignited over a Bunsen burner for about fifteen minutes, again cooled in a desiccator, and reweighed. The difference gives the weight of ash-free fibrin.

Micro Kjeldahl determination of fibrin precipitate

The clot obtained by Cullen and Van Slyke's method from 1 cc. of plasma is transferred to a dry filter. While filtering insert into the jelly a slender glass rod with a pointed end and rotate gently (29). All the fibrin will usually stick to the rod. Transfer the fibrin to a dry filter paper and slip it off the rod. If any bits of fibrin have remained on the first filter they are picked up with the tip of the rod and transferred to the second. The clot is pressed in the second filter paper to remove adherent fluid as completely as possible.

For micro Kjeldahl determination the clot is transferred to a proper test tube or micro Kjeldahl flask and analyzed as described for proteins in the nitrogen chapter.

Colorimetric determination of fibrin precipitate. Wu (29, 30)

The procedure of Wu and Ling (30) is used. The Folin colorimetric phenol reagent is used, and the color developed is presumably due to the tyrosine group in the fibrin. The later Folin and Ciocalteu phenol reagent employed above for albumin and globulin can not at present be used for fibrin because the factor for it expressing the mg. of fibrin that give color equivalent to 1 mg. of tyrosine has not yet been determined.

Colorimetric reagents.

1 N sulfuric acid.

2.5 N sodium hydroxide.

0.25 N sodium hydroxide.

The original Folin phenol reagent, which is prepared as follows: In a 1.5 liter flask dissolve 100 grams of sodium tungstate, $Na_2WO_4 \cdot 2H_2O$, and 25 grams of sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$, in 700 cc. of water. Add 50 cc. of 85 per cent phosphoric acid (the syrupy phosphoric acid of commerce) and 100 cc. of concentrated HCl. Boil with a reflux condenser for eight hours and make up to 1 liter.

A standard tyrosine solution prepared by dissolving 50 mg. of tyrosine in 250 cc. of 0.1 N hydrochloric acid.

Colorimetric procedure. **The fibrin clot, freed from adherent solution as above described for the micro Kjeldahl determination, is transferred to a 15-cc. centrifuge tube. Four cubic centimeters of the 0.25 N NaOH are added and the tube is heated in a water bath until the fibrin has all dissolved, leaving calcium oxalate in suspension. Ten cubic centimeters of water added and the mixture is centrifuged to remove the calcium oxalate. The supernatant solution is transferred to a 25-cc. volumetric flask, 0.5 cc. of the phenol reagent is added, then water to about 20 cc., 1 cc. of the 2.5 N sodium hydroxide, and finally water again to the 25-cc. mark. The solution is mixed and let stand an hour for the color to develop. At the same time a standard is prepared by measuring 1 cc. of the tyrosine solution into a 25-cc. flask, adding 0.5 cc. of phenol reagent, water to 20 cc., etc. At the end of the hour unknown and standard are compared in a colorimeter.**

Since the 0.2 mg. of tyrosine in the standard gives as much color as 2.26 mg. of fibrin, the fibrin content of the plasma is calculated as follows:

$$\frac{S}{U} \times 0.226 = \text{grams of fibrin per 100 cc. of plasma.}$$

where U and S represent colorimetric readings of unknown and standard, respectively, and the fibrin is from a 1 cc. sample of plasma.

DETERMINATION OF TOTAL PROTEIN IN CEREBROSPINAL FLUID, AND IN TRANSUDATES AND EXUDATES

The micro Kjeldahl or Wu and Ling colorimetric procedure described above for urine is applied. The amount of cerebrospinal fluid taken is

usually 5 cc. Of transudates and exudates from 0.3 to 2.0 cc. is taken, depending upon the protein content. Since the amount of non-protein nitrogen is relatively small compared with that in the urine, one reprecipitation only is necessary before the final analysis.

BIBLIOGRAPHY

1. AUTENRIETH, W., AND MINK, F.: Ueber Kolorimetrische Bestimmungsmethoden: die quantitative Bestimmung von Harneiweiss. *Münch. Med. Woch.*, 1915, **62**, 1417.
2. AUTENRIETH, W.: Ueber kolorimetrische Bestimmungsmethoden: Die Bestimmung von Serumalbumin und Globulin im Harn, in der Aszitesflüssigkeit und im Blutserum. *Münch. med Woch.*, 1917, **64**, 241.
3. BIERRY, H., AND VIVARIO, R.: Dosage des albumins globales du plasma sanguin. *Compt. rend. soc. biol.*, 1923, **89**, 13.
4. CULLEN, G. E., AND VAN SLYKE, D. D.: Determination of the fibrin, globulin and albumin nitrogen of blood plasma. *J. Biol. Chem.*, 1920, **41**, 587.
5. FOLIN, O., AND CIOCALTEU, V.: On tyrosine and tryptophane determinations in proteins. *J. Biol. Chem.*, 1927, **73**, 627.
6. FOLIN, O., AND DENIS, W.: The quantitative determination of albumin in urine. *J. Biol. Chem.*, 1914, **18**, 273.
7. FOLIN, O., AND DENIS, W.: Metabolism in Bence-Jones proteinuria. *J. Biol. Chem.*, 1914, **18**, 277.
8. FOSTER, D. P., AND WHIPPLE, G. H.: Blood fibrin studies. I. An accurate method for the quantitative analysis of blood fibrin in small amounts of blood. *Am. J. Physiol.*, 1922, **58**, 365.
9. GRAM, H. C.: A new method for the determination of the fibrin percentage in blood and plasma. *J. Biol. Chem.*, 1921, **49**, 279.
10. GREENBERG, D. M.: The colorimetric determination of the serum proteins. *J. Biol. Chem.*, 1929, **82**, 545.
11. GUILLAUMIN, C. O.; WAHL, R., AND LAURENCIN, M. L.: Sur le dosage des albumines sériques. Comparison de quelques résultats obtenus par pesée, par azotométrie, et par réfractométrie. *Bull. Soc. Chim. Biol.*, 1929, **11**, 387.
12. HAMMARSTEN, O.: Ueber das Paraglobulin. *Pflüger Arch. Physiol.*, 1878, **17**, 413.
13. HILLER, A.: Determination of albumin and globulin in urine. *Proc. Soc. Exp. Biol. Med.*, 1927, **24**, 385.
14. HILLER, A.; MCINTOSH, J. F., AND VAN SLYKE, D. D.: The excretion of albumin and globulin in nephritis. *J. Clin. Invest.*, 1927, **4**, 235.
15. HOWE, P. E.: The use of sodium sulfate as the globulin precipitant in the determination of proteins in blood. *J. Biol. Chem.*, 1921, **49**, 93.
16. HOWE, P. E.: The determination of proteins in blood—a micro-method. *J. Biol. Chem.*, 1921, **49**, 109.
17. HOWE, P. E.: The relative precipitating capacity of certain salts when applied to blood serum or plasma and the influence of the cation in the precipitation of proteins. *J. Biol. Chem.*, 1923, **57**, 241.
18. HOWE, P. E.: The determination of fibrinogen by precipitation with sodium sulfate compared with the precipitation of fibrin by the addition of calcium chloride. *J. Biol. Chem.*, 1923, **57**, 235.

19. LINDER, G. C.; LUNDSGAARD, C., AND VAN SLYKE, D. D.: The concentration of the plasma proteins in nephritis. *J. Exp. Med.*, 1924, **39**, 887.

20. MOORE, N. S., AND VAN SLYKE, D. D.: The relationships between plasma specific gravity, plasma protein content, and edema in nephritis. *J. Clin. Invest.*, 1930, **8**, 337.

21. NEUHAUSEN, B. S., AND RIOCH, D. M.: The refractometric determination of serum proteins. *J. Biol. Chem.*, 1923, **55**, 353.

22. REISS, E.: Der Brechungkoeffizient der Eiweisskörper des Blutserums. *Beitr. chem. Physiol. Path.*, 1904, **4**, 150.

Eine neue Methode der quantitativen Eiweissbestimmung. *Arch. exp. Path. Pharm.*, 1904, **51**, 18.

Untersuchungen der Blutkonzentration des Säuglings. *Jahrb. Kinderh.*, 1909, **70**, 311.

23. ROBERTSON, T. B.: A micro-refractometric method determining the percentage of globulin and albumin in very small quantities of blood serum. *J. Biol. Chem.*, 1915, **22**, 233.

24. SHEVKY, M. C., AND STAFFORD, D. D.: A clinical method for the estimation of protein in urine and other body fluids. *Arch. Int. Med.*, 1923, **32**, 222.

25. STARLINGER, W.: Über die Methodik der quantitativen Bestimmung des Fibrinogens. *Biochem. Z.*, 1923, **140**, 203.

26. STARLINGER, W., AND HARTL, K.: Über die Methodik der quantitativen Bestimmung des Fibrinogens. III. *Biochem. Z.*, 1925, **157**, 283.

27. TAYLOR, A. E., AND MILLER, C. W.: Studies in Bence-Jones proteinuria. I. *J. Biol. Chem.*, 1916, **25**, 281.

28. TSUCHIYA, I.: Eine neue volumetrische Eiweissbestimmung mittels der Phosphorwolframsäure. *Centr. inn. Med.*, 1908, **29**, 105.

29. WU, H.: A new colorimetric method for the determination of plasma proteins. *J. Biol. Chem.*, 1922, **51**, 33.

30. WU, H., AND LING, S. M.: Colorimetric determination of proteins in plasma, cerebrospinal fluid, and urine. *Chinese J. Physiol.*, 1927, **1**, 161.

CHAPTER XXII

Blood Volume

DISCUSSION

Welcker (29) made the first careful study of the blood volume of experimental animals by exsanguinating them completely, a procedure which had already been applied to human beings (criminals) by Lehmann and Weber (15). Although similar procedures have been employed by subsequent investigators in physiological studies, they are obviously unsuitable for clinical purposes. For such purposes methods have been devised which depend on two general principles.[1]

1. A known amount of some foreign substance, such as a colloidal dye or carbon monoxide, which will not diffuse out of the circulation to a significant extent in the time required to mix with the entire blood stream, is admitted to the latter. After mixture is complete the concentration of the foreign substance is determined, and from the result the volume of blood with which the substance has been diluted is calculated.

2. The change of concentration of some normal blood constituent may serve, under limited conditions, as a measure of *changes* in blood volume, but is of no value for the determination of the absolute volume of the circulating blood.

Both types of methods may again be subdivided into: (a) those that measure directly the number or volume of the circulating red blood cells and (b) those that measure the volume of the plasma.

General requirements of such methods are that: 1, within the time limit of the experiment the substances dissolved in blood to measure its volume must not leave the circulation in significant and undetermined quantities; 2, such substances must be uniformly dispersed throughout the blood stream; 3, they must remain confined to that phase of the blood—i.e., plasma or cells—which is to be estimated, or be uniformly distributed in both phases; and 4, the sample of blood withdrawn must be fairly representative of that in the whole circulating system.

It is impossible to claim that any method yet devised has been proven to meet completely and quantitatively all these requirements. Both the dye method and the carbon monoxide method, however, appear to approach the

[1] For a review of the literature on blood volume methods up to 1919 see Salvesen (22).

requirements with sufficient constancy to give results which serve as useful approximations.

These two types of methods do not indicate the same blood volumes. The dye method measures directly the volume of the plasma, since the dye is dissolved in this medium. The volume of whole blood is calculated from the plasma volume by multiplying the latter by the ratio, 100 : volume per cent plasma in blood, found by hematocrit. By the dye method an average plasma volume in normal men has been found of approximately 50 cc. per kilo body weight (11). From this, and the mean normal plasma proportion of 54 volumes per cent, the mean blood volume is calculated at about 93 cc. per kilo by the dye method. Results from injection of other colloids, hemoglobin (6, 14), gelatin (18), or gum acacia (18, 19), agree with those by the vital red method. On the other hand, the mean total blood volume found by the carbon monoxide method has been 60 or 70 cc. per kilo (3, 22). The source of this difference has been the cause of much work and discussion.

It has been especially studied by Whipple and Smith and their collaborators (1, 2a, 4, 10, 23, 24, 25, 26). The source of error most readily suspected of causing high results is escape of the dye from the circulation. Yet these investigators found that different dyes gave the same results, and the curves of their rate of diminution in the blood with time indicated that if the rate of escape in the first four minutes, before the blood was drawn for the first analysis, was not faster than during the next four minutes, the error from this source was slight. Also when dissolved hemoglobin instead of dye was injected, the plasma volume calculated from its dissolved hemoglobin content (14) was the same found by the dye methods. The decreases in plasma volume found by the dye method after controlled hemorrhages (2a) approximated the volumes of plasma withdrawn. The explanation offered by Smith, Arnold, and Whipple (26) for the high blood volumes obtained the dye, compared with the CO method, was that the ratio, plasma volume : cell volume, is in reality greater in much of the blood in the body than in the sample withdrawn from a peripheral vein for analysis. The greater part of the circulating blood is in the capillaries and small vessels. Smith, Arnold, and Whipple pointed out that microscopic observation indicated the presence next the walls of the small vessels of a ring of plasma practically free from corpuscles, and that this plasma ring might constitute as much as 40 per cent of the blood in such vessels. Hence these authors believed that the dye methods indicated correctly the volume of blood *plasma* in the body, but that when the volume of blood cells or total blood was calculated from the plasma volume by means of a cell : plasma ratio equal to that in an analyzed venous sample, the cell or blood volumes so estimated were grossly

too high. Later results of Smith (25), however, indicate that part of the reason for the high plasma volume obtained by the dye methods may in fact be escape of the dye from the circulation. Smith found that in a few minutes enough dye had entered the lymph to make a larger error than the 5 per cent which he, Arnold, and Whipple (26) had estimated by assuming that escape of dye from the circulation was no greater in the first four minutes than in the second four minutes. To judge from Smith's studies of the lymph, there may be a sudden passage of dye into the lymph during the first minutes after injection, and relatively slow passage thereafter.

There appears to be no reason to ascribe any error to the assumption that dye is evenly mixed throughout the blood stream four minutes after its injection. Keith, Rowntree and Geraghty (11) and Smith (24, 25) have shown that the concentration of vital red in the circulating blood changes but little between the fourth and tenth minutes after its injection.

It is possible that the inherent errors of the dye method are exaggerated in certain physiological conditions, as Lamson (12, 13) has suggested. He found paradoxical differences in the variations of hemoglobin, cell volume, and dye concentrations under certain conditions. These differences may at least in part be due, as Smith (25) has pointed out, to swelling or contraction of the cells in response to chemical reactions.

We may conclude that volumes of plasma and blood estimated by dye methods are probably too high, but that if every precaution is taken to avoid the introduction of additional extraneous sources of error and the technique is carried out in the standard manner prescribed by Hooper, Smith, Belt and Whipple (10), results can be obtained which give an indication of the variations in health and disease.

The carbon monoxide method was first applied by Gréhant and Quinquaud (8), who determined the concentration of carbon monoxide in the blood after inhalation of known amounts of the gas.

The method is applied with the assumption that, because of the great affinity of blood hemoglobin for carbon monoxide, all of the gas remains in the circulating red blood cells. This assumption was questioned by Whipple and his collaborators (1). They pointed out that the body contains hemoglobin outside the circulation, particularly in the bone marrow and in the form of myohemoglobin in the red muscles. The myohemoglobin is identical with blood hemoglobin in many, perhaps all, respects. It combines with the same proportion of oxygen (4a). It also combines with carbon monoxide. Whipple (30) finds that in dogs the myohemoglobin is present in amounts to combine with from 10 to 80 per cent as much oxygen as the blood hemoglobin, the greatest amounts of myohemoglobin being present in

the muscles of animals which were fit from training, the least in those which had been confined. It is obvious that when the tissues contain such amounts of hemoglobin, carbon monoxide which enters the blood must, if given sufficient time, redistribute itself between blood and tissues in proportion to the amounts of hemoglobin in each. The question of importance, in so far as the carbon monoxide blood volume method is concerned, is: Does the passage of CO from blood to tissues occur with sufficient *rapidity* to decrease significantly the amount of the gas in the blood during the few minutes required for a blood volume determination? A decisive

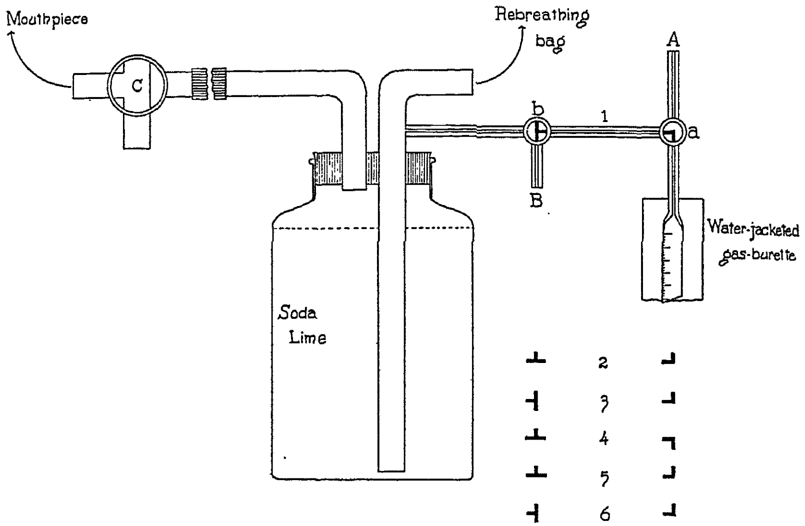


FIG. 84. Diagram of apparatus for the administration of carbon monoxide in the determination of blood volume. From Chang and Harrop (3).

answer to this question might be given by permitting animals to inhale CO for several minutes, and then killing them, immediately exsanguinating the muscles, and analyzing them for the CO compound of myohemoglobin. Such experiments have not been done, and in their lack one must draw what appear to be the most probable conclusions from other facts and from the known behavior of carbon monoxide combined with blood hemoglobin.

From the following considerations it appears improbable that the shift to the muscles and other extracirculatory depots of hemoglobin is significant in the few minutes required for a determination. Van Slyke and Robbins (27) injected intravenously into dogs blood saturated with carbon monoxide

and withdrew samples of venous blood at intervals beginning three minutes after the injection. Any decrease in CO content of the circulating blood after mixture with the injected sample would be the sum of two losses, to air in the lungs and to muscle and other hemoglobin in tissue depots. The loss through both paths in the seven minutes after the first three-minute samples were drawn was only a few per cent of the injected CO. It appears that if a marked passage of CO from the blood to the tissues occurs during the first three minutes it must occur by means of a diffusion incomparably faster than that to both lung air and tissues during subsequent minutes. In other words, CO must distribute itself between blood and tissues so quickly that almost an instantaneous equilibrium is attained. This would require CO to dissociate from its firm combination with blood hemoglobin and pass into the tissues with infinitely greater speed than it dissociates and passes from the blood into the alveolar air during circulation through the lungs. Such a rapid dissociation of carboxyhemoglobin, and such a relatively greater loss to the tissues than to the air in the lungs, where the structure is especially adapted to rapid gas exchange, seems practically impossible.

An idea of the possible maximum rate of diffusion of CO from blood into tissues of man may be gained from an experiment of Chang and Harrop (3). They had subjects breath air containing CO from a closed system, such as is shown in figure 84, for twenty minutes, during which practically all of the CO was absorbed by the subjects. Then, while still breathing air from the same system, so that loss of CO from the lungs was excluded, the subjects exercised vigorously on a stationary bicycle for ten minutes. This procedure should open up the capillaries of the large muscles and stimulate circulation through them in such a way that the diffusion of CO into the muscle tissue would be several times faster than in the resting subject. The average diminution of the CO content of the blood during the exercise was only 5 per cent.

Smith, Arnold and Whipple (26) were inclined to believe that a quick distribution of CO between blood and tissues did occur. They found that when the amount of hemoglobin in the bodies of dogs calculated by the CO method was compared with the amount that could be obtained from the blood by exsanguinating the same animals and washing out their vessels by perfusion, the blood hemoglobin thus regained varied from 67 to 100 per cent of that calculated by the CO method, and averaged 85 per cent. When to the hemoglobin from the drawn blood was added that which could be extracted from the tissues and bone marrow, it brought the average up to 95 per cent of that found by the CO method. Hence Smith, Arnold, and Whipple (26) believed that the CO method measured the total tissue + blood hemoglobin rather than only the blood. In view of the variability of the results, however, and of the considerations advanced above, it appears more probable that the 15 per cent average deficit hemoglobin found in the blood, compared with that calculated by the CO method, was attributable to other factors than sudden diffusion of CO from blood to tissues. Any failure to obtain and determine 100 per cent of the blood hemoglobin by the exsanguination method would cause a discrepancy, and it appears not impossible that the deficit found in the hemoglobin of the blood may have been due to unavoidable experimental difficulties in obtaining it all.

In so far as one can judge, the carbon monoxide method appears to be without serious sources of error for determination of the amount of circulating *cells and hemoglobin.* The extent of its accuracy for the volumes of plasma and whole blood depends upon the constancy of the hemoglobin content of blood in the large and small vessels. Smith, Arnold and Whipple (26) have advanced serious arguments (quoted above in discussing the dye method) to show that the blood in the small vessels contains much more plasma and less cells than the blood of the large veins tapped for analyses. If these arguments hold, it is probable that volumes of plasma, and hence of whole blood, estimated by the carbon monoxide and hematocrit method are too low; but whether by a significant extent appears at the moment uncertain.

THE VITAL RED METHOD FOR THE DETERMINATION OF BLOOD AND PLASMA VOLUME. KEITH, ROWNTREE, AND GERAGHTY (11)

These authors first employed vital red for the determination of plasma volume in 1915 (11). They found that this dye was non-toxic, that it was only slowly removed from the circulation, and that it lent itself well to colorimetric estimation.

During the war the supply of vital red in this country became exhausted. This led Whipple and his associates (4, 10, 24) to seek a substitute. They found that hemoglobin (14) and several dyes besides vital red were satisfactory for plasma volume estimation. The best of these were brilliant vital red and a blue azo dye, ortho toluidine combined with 2 molecules of 1.8-amido-2.4-sulphonic acid, T-1924 of their series (4). The latter is perhaps the best of all the dyes investigated. Because of its blue color it does not mask hemolysis as the red dyes do. It is, however, not easy to obtain and therefore has not been employed by others. Brilliant vital red is easy to procure and, if due precautions are taken to prevent hemolysis, is eminently satisfactory.

Hooper, Smith, Belt and Whipple (10) also modified the method of Keith, Rowntree and Geraghty by mixing the blood sample with an isotonic solution of oxalate instead of the powdered salt to prevent coagulation. The latter causes the cells to shrink and therefore disturbs the proportions of cells and plasma and yields high relative values for plasma volume.

Franke and Benedict (6) have, like Lee and Whipple (14), found that the dye technique could be applied to animals with the use of hemoglobin solutions for injection in place of dye solutions. Franke and Benedict used a solution prepared by laking centrifuged cells in about 4 volumes of water and then adding enough NaCl to make the solution isotonic. Of this they injected 1 cc. for each kilo body weight. The method has apparently not been applied to human subjects, and might be dangerous unless the hemoglobin solutions were made from human cells from individual belonging to the same blood group as the subject.

Precautions which must be observed

Throughout the procedure the production of venous stasis must be avoided. A tourniquet may not be used. Instead, momentary gentle

pressure may be applied to the arm just above the elbow for a few seconds immediately preceding each venous puncture and must be removed as soon as the needle has entered the vein. The extremities should be warm at the time of the experiment. Lindhard (17) recommends moderate exercise between the two venous punctures to accelerate the circulation and promote distribution of the dye. He has shown that this measure greatly diminishes the concentration of dye in the blood. This he interprets as evidence of more complete dissemination of the dye. It may, however, indicate escape of the dye from the vessels. At any rate exercise has not been adopted by others as part of the technique of blood volume determination, and causes values far higher than any that have been previously obtained by any blood volume method. To obtain results comparable to others in the literature, exercise should therefore be avoided. Lindhard (17) claims that if dye injections are repeated often on the same subject it is found that the subject develops a tolerance for the dye which enables him to remove it from the blood more rapidly than usual. When this tolerance has developed the method is no longer applicable. Smith (23) on the other hand, experienced no difficulty with repeated determinations in dogs.

The interval between the injection of dye and the removal of the blood for analysis must be not less than four minutes and as little as possible greater than four minutes. Care must be taken that all the dye enters the vein.

Dye, saline, and other solutions which are intended for intravenous injection should be prepared from water distilled not more than twenty-four hours before the determination, to prevent production of "water or salt reactions," and must be carefully sterilized by boiling or autoclaving.

Special solutions and apparatus required

Freshly distilled water (see above), sterilized by boiling for ten minutes.

Normal saline solution. Nine grams of NaCl diluted to 1 liter with freshly distilled water. This should be boiled ten minutes to sterilize it, cooled again to room temperature, and then diluted to volume with the sterile boiled water, with precautions against bacterial contamination.

A 1 per cent solution of brilliant vital red (Evans' for blood volume estimation[2]) in freshly distilled water. This should be sterilized, like the saline, by boiling and then brought back to volume with sterile distilled water.

A $\frac{1}{267}$ *per cent standard brilliant vital red solution* made by diluting 0.75 cc. of the 1 per cent solution to 200 cc. with water.

[2] It is essential to use a high grade preparation suitable for intravenous use, if untoward reactions are to be avoided.

Isotonic 1.6 per cent solution of sodium oxalate (10). Dissolve 1.6 grams of sodium oxalate in water and dilute to 100 cc. Place 10 cc. of the solution in a test tube and add 2 drops of 0.07 per cent phenol red. If necessary titrate to a pH of 7.3 ± 0.2 with 0.1 N oxalic acid or sodium hydroxide. Add a proportional amount of the acid or alkali to the remaining 90 cc. of oxalate. For example, if 0.1 cc. of 0.1 N oxalic acid is required to neutralize 10 cc. of oxalate add 0.9 cc. to the remaining 90 cc. of solution.

Accurately calibrated centrifuge tubes.

Syringes, 20-cc., accurately calibrated.

Procedure

A needle is inserted into one of the antecubital veins without stasis and a sample of blood is withdrawn. Through the same needle a known volume of the sterile 1 per cent vital red solution (about 1 cc. for each 5 kilos of body weight) is introduced and washed in with a small amount of sterile saline. The needle is then withdrawn from the vein.

After an interval of exactly four minutes a second sample of blood is taken from a vein of the opposite arm.

Each sample of blood, as soon as it is taken, is transferred to a centrifuge tube containing enough isotonic oxalate solution to make a mixture of about 1 part oxalate to 4 parts of blood. The volume of oxalate solution used must be accurately measured.

The two centrifuge tubes are now whirled for thirty to forty-five minutes at 2000 to 2500 r.p.m. The volume of the red cells and the total volume of blood + oxalate in the tube containing the dyed blood are read off. As an alternative the cell volume may be determined in an ordinary hematocrit (see p. 73).

Two cubic centimeters of the dye-colored plasma are removed and diluted with 4 cc. of 0.9 per cent NaCl solution. This mixture is compared in the colorimeter with a standard prepared by mixing equal volumes of 0.9 per cent NaCl solution, standard vital red solution, and uncolored plasma.

Calculation of results

$$\frac{267\ D\ C\ U}{S} = \text{cubic centimeters of plasma volume in the body}$$

U and S are readings of unknown and standard respectively in the colorimeter. D = the cubic centimeters of 1 per cent vital red solution injected. C is the ratio

$$\frac{\text{Cubic centimeters of plasma}}{\text{Cubic centimeters of plasma} + \text{oxalate}}$$

in the centrifuge tube.

$$\text{Blood volume} = \text{plasma volume} \times \frac{100}{\text{per cent plasma in blood}}$$

Per cent plasma in blood is the observed percentage of plasma + oxalate solution, measured in the centrifuged oxalated blood, multiplied by C.

The formula is derived as follows.

The concentration of dye in the plasma is inversely proportional to the volume of plasma with which the injected dye is diluted. Therefore:

$$(\text{Cubic centimeters of plasma volume}) : D = 1 : (\text{per cent dye in plasma})$$

$$\text{Cubic centimeters of plasma volume} = \frac{D}{\text{per cent dye in plasma}}$$

$$\text{Per cent dye in oxalate plasma} = \frac{1}{267} \times \frac{S}{U}.$$

$$\text{Per cent dye in circulating plasma} = \frac{1}{267} \times \frac{S}{U} \times \frac{1}{C}.$$

Hence,

$$\text{Cubic centimeters of plasma volume} = 267\ D\ U\ C$$

Example: Fifteen cubic centimeters of dye are injected. Standard and unknown readings are 20 and 25 mm. respectively. Two cubic centimeters of oxalate and 8 cc. of blood are mixed in the centrifuge tube, and 7 cc. of clear fluid are found over the blood cells in the tube after centrifugation. Of the 7 cc. of clear solution, 2 cc. are added oxalate solution, and the remaining 5 cc. represent the plasma volume. Hence $C = \frac{5}{7}$, and the plasma content of the blood was $\frac{5}{8} \times 100 = 62.5$ volumes per cent. From these data the calculations are made:

$$\text{Volume of plasma in body} = \frac{267 \times 15 \times \frac{5}{7} \times 25}{20} = 3580 \text{ cc.}$$

$$\text{Volume of blood in body} = 3580 \times \frac{100}{62.5} = 5780 \text{ cc.}$$

Results are given to only 3 significant figures, because the accuracy of the method does not justify expressing the fourth figure.

THE CARBON MONOXIDE METHOD

This method, devised by Gréhant and Quinquaud (8), was later applied by Haldane and Smith (9) and by Douglas (5) to the elucidation of physiological problems. For the determination of CO in blood Haldane and Smith and Douglas employed carmine titration, difficult to perform accurately. The accuracy of the procedure was enhanced by introduction of the gasometric blood CO method by Van Slyke and Salvesen (28). However, the sensibility of the analytical method still remained so low that accurate determinations of blood volume required that as much as one-third of the blood hemoglobin be saturated with CO. Later refinements of analytical technique by Van Slyke and Robbins (27) and Chang and Harrop (3) permit the attainment of an equal degree of accuracy when only one-tenth of the blood hemoglobin is saturated with CO. The test is thereby rendered useful for clinical purposes.

For animal experimentation Van Slyke and Robbins (27) have described a technique in which a known amount of blood saturated with CO gas is injected intravenously. For clinical work, however, inhalation appears to be a preferable method of administration.

Procedure for administration of carbon monoxide gas by Chang and Harrop's (3) Modification of Gréhant and Quinquaud's method

The *apparatus* is illustrated schematically in figure 84. It consists of a rubber rebreathing bag into which the patient breathes through a rubber mouth piece (a close-fitting mask may be used for unconscious subjects) and a glass-connecting tube. Between the bag and the mouthpiece is interposed a soda-lime container to remove carbon dioxide. This bottle may be placed in an ice jacket to precipitate excessive moisture. The bag is equipped with a side arm through which measured amounts of carbon monoxide can be introduced from a gas burette and oxygen can be admitted from a gas cylinder to replace that removed from the bag by the subject. That complete mixture with air in the lungs may be quickly obtained, the total amount of gas in the bag is kept as small as possible, so that at the end of inspiration it is nearly collapsed. Oxygen is admitted from a pressure tank equipped with a needle valve in amounts just sufficient to balance removal. The oxygen concentration in the gas breathed should not exceed 30 per cent. A greater concentration of oxygen retards the combination of blood hemoglobin with CO. In the apparatus of Chang and Harrop the total volume of gas in the system at the moment of complete normal expiration did not usually exceed 2500 cc. A side tap placed in the connection between bag and absorber permits the removal of a gas sample just before

the conclusion of the experiment, to determine the concentration of CO in the bag.

Carbon monoxide is prepared and stored by the method described on page 344 in the chapter on gasometric methods. The gas should be analyzed from time to time to test its purity. Such analyses can be made in a Haldane or Van Slyke-Neill gas analysis apparatus, either by combustion (as described for hydrogen) or by absorption with cuprous chloride solution. They can be carried out with sufficient accuracy by shaking a measured amount of gas in a Hempel pipette with the cuprous chloride solution and measuring the unabsorbed residue.

Details of Procedure

The whole system shown in figure 84 is first washed out with air to remove residual traces of carbon monoxide. This can be accomplished with use of suction if desired.

After the washing a small amount of air is introduced into the rebreathing bag through stopcock *b* or through the breathing tube as desired. Valve *c* is then turned to connect the mouth piece with the room air; cocks *b* and *a* are set in position *1*.

CO is introduced into the burette through *B* from the CO reservoir through an interposed three-way stopcock. This allows the burette connections to be washed out with the gas before the burette is filled. When the burette is full and adjusted to atmospheric pressure the reservoir stopcock is connected with the outside air and stopcocks *a* and *b* are in position *2*. The CO is then washed from the connecting tubes by expelling air from the rebreathing bag first through *B* and then through *A*.

The CO reservoir is now removed and the oxygen cylinder is connected to *A*. With the stopcocks set in position *3*, the required amount of air is introduced into the rebreathing bag through *B* by water displacement from coupled aspirator bottles, and stopcock *b* is turned to position *4*. After the volume of CO in the burette has been read under atmospheric pressure *a* is set in position *4* and the desired amount of CO is driven into the bag by raising the leveling bulb. *a* is now turned to position *5*, a few cubic centimeters of oxygen are admitted to drive the residual gas from the connections into the bag, and the volume of CO remaining in the burette is read under atmospheric pressure.

For an adult of average size about 100 cc. of CO are required, with proportional variation for children and for adults who deviate greatly

from the average size. The observed volume of CO introduced is reduced to standard conditions by means of the factors on p. 129.

The mouthpiece is now comfortably adjusted to the mouth of the patient and the nose-clip is attached. After a short preliminary period to permit the patient to become accustomed to the apparatus, cock *c* is connected with the rebreathing bag. Rebreathing is continued for twenty minutes, during which oxygen is slowly admitted as it is required from the cylinder. Just before the close of the period the oxygen is shut off and the patient is allowed to rebreathe the air in the system. At the end of the twenty minutes cock *c* is again connected with the outside air and the patient is disengaged.

The amount of unabsorbed ***residual CO*** left in the bag and the lungs must be ascertained and subtracted from the amount of CO introduced into the system, in order to calculate the amount of CO absorbed into the blood. Without, as a rule, causing significant error in the blood volume found, one may assume that the residual CO is 1 per cent of the CO introduced. (If the residual CO were twice as much, it would make an error of only 1 per cent in calculating the absorbed CO and the blood volume.)

Theoretically a more precise procedure is to analyze the gas in the bag for CO, and multiply the CO concentration found by the total gas volume in the lungs and apparatus at the moment when the experiment was ended. The CO content of the gas in the bag may be determined by one of the methods given in Chapter 3. Methods for estimating both the gas volume in the apparatus and that in the lungs are given in the chapter on lung volume.

Just before the close of the rebreathing period a sample of blood is taken from a vein of the arm by the anaerobic technique of Austin et al. (see p. 53) with the precautions against stasis described under the vital red method. The blood may be defibrinated or oxalated. Samples are analyzed for carbon monoxide by the method of Van Slyke and Robbins or of Sendroy and Liu (see gasometric methods, p. 332).

If it is desired to estimate the volumes of cells and serum separately the blood may be defibrinated anaerobically and the cell volume determined by means of the hematocrit. Or oxalate may be used as anticoagulant. In this case a measured volume of blood is mixed in a second tube with a known volume of neutral, isotonic (1.6 per cent), solution of sodium oxalate, as described for the dye method, and the mixture is used for hematocrit determinations.

Calculation

$$\text{cubic centimeters of CO introduced} - \text{cubic centimeters of residual CO} = \text{cubic centimeters of CO absorbed}$$

$$\frac{100 \times \text{cubic centimeters of CO absorbed}}{\text{volume per cent of CO in blood}} = \text{cubic centimeters of blood volume in body}$$

$$\text{cubic centimeters of blood volume} \times \frac{\text{per cent cells in blood}}{100} = \text{cubic centimeters of cell volume}$$

$$\text{cubic centimeters of blood volume} - \text{cubic centimeters of cell volume} = \text{cubic centimeters of plasma volume.}$$

ESTIMATION OF BLOOD VOLUME CHANGES FROM CHANGES IN CELL OR PROTEIN CONTENT

For the determination of changes in blood volume, variations in the concentration of different normal blood constituents have been employed. The constituents most commonly employed are hemoglobin, red blood cell volume, serum or plasma proteins, and total solids. Such methods are, of course, of limited value. Although most of the substances enumerated do not, under ordinary circumstances traverse the vessel walls, their concentration in the blood is not dependent solely upon the volume of the blood.

Changes in hemoglobin concentration and in cell count (2) of the blood serve, with a degree of accuracy at present quite uncertain, to indicate changes in the volume of total circulating blood. The possibility has already been discussed that the concentration of red cells and hemoglobin may be less in the blood of the small vessels than in that of the large ones. Perhaps because of variations in the distribution of the blood between the two types of vessels, the hemoglobin concentration of blood from the peripheral vessels appears to be capable of quick changes amounting to as much as 10 per cent (see hemoglobin chapter of volume I, p. 549). Unless control tests carried out on the same subject under the conditions of the observation show no tendency to such spontaneous disturbances, it is difficult to attribute much accuracy to hemoglobin or cell content changes as measures of blood volume changes.

Changes in the volumes per cent of red cells determined by hematocrit are even less reliable as measures of blood volume variations. In addition to the factors that cause spontaneous variation in hemoglobin concentration, the cell volume is affected by changes in the pH and osmotic pressure of the plasma, which cause the cells to swell or contract.

Plasma proteins aid only in estimation of changes of plasma volume and may not be used, as they too frequently have been, to measure variations of total blood volume. Under normal conditions it is justifiable to assume that the vessel walls and blood cell membranes are impervious to protein, but in certain circumstances it is possible and even probable that proteins escape from the capillaries (7). Until more is known of the site and manner of production and destruction of these proteins it is impossible to evaluate the importance of such factors in the causation of protein changes.

All these methods for the determination of relative blood or plasma volume are applicable only to the study of rapid changes. The intervals between determinations must be short enough to preclude the effect of regenerative and destructive processes.

BIBLIOGRAPHY

1. Arnold, H. R.; Carrier, E. B.; Smith, H. P., and Whipple, G. H.: Blood volume studies. V. The carbon monoxide method—its accuracy and limitations. *Am. J. Physiol.*, 1921, **56**, 313.

2. Ashby, W.: Blood volume. I. A method for determinating whole blood volume based on the circulating corpuscle volume. *Arch. Int. Med.*, 1925, **35**, 516.
   II. A comparison between total blood volumes determined by plasma volume methods and by a new corpuscle volume method. *Ibid.*, 632.
   III. Apparent changes in blood volume induced by transfusion, and their bearing on methods of determining blood volume by means of the degree of change in a constituent of the blood, following transfusion of a known amount of that constituent. *Ibid.*, 641.
   IV. Diurnal fluctuations in blood volume and change incident to transfusion reaction. *Ibid.*, 726.
   V. The effect of treatment on the blood volume of patients with pernicious anemia. *Ibid.*, 733.
   VI. The relationship between blood volume, total corpuscle content and alkaline reserve in cases of pernicious anemia. *Ibid.*, **36**, 24.

2a. Carrier, E. B.; Lee, F. W., and Whipple, G. H.: Determination of plasma and hemoglobin volumes after unit hemorrhages under controlled experimental conditions. *Am. J. Physiol.*, 1922, **61**, 138.

3. Chang, H. C., and Harrop, G. A., Jr.: The determination of the circulating blood volume with carbon monoxide. *J. Clin. Invest.*, 1928, **5**, 393.

4. Dawson, A. B.; Evans, H. M., and Whipple, G. H.: Blood volume studies. III. Behavior of large series of dyes introduced into the circulating blood. *Am. J. Physiol.*, 1920, **51**, 232.

4a. Dirken, H. N. J., and Mook, H. W.: The oxygen combining power of muscle hemoglobin. *J. Physiol.*, 1930, **69**, 210.

5. Douglas, C. G.: The determination of the total oxygen capacity and blood volume at different altitudes by the carbon monoxide method. *J. Physiol.*, 1910, **40**, 472.

6. Franke, E., and Benedict, S. R.: A method for the determination of blood volume. *J. Lab. Clin. Med.*, 1921, **6**, 618.

7. GASSER, H. S.; ERLANGER, J., AND MEEK, W. J.: Studies in secondary traumatic shock. IV. The blood volume changes and the effect of gum acacia on their development. *Am. J. Physiol.*, 1919, **50**, 31.

8. GRÉHANT, M., AND QUINQUAUD, E.: Mesure du volume de sang contenu dans l'organisme d'un Mammifère vivant. *Compt. rend. Acad. Sci.*, 1882, **94**, 1450.

9. HALDANE, J., AND SMITH, J. L.: The mass and oxygen capacity of the blood in man. *J. Physiol.*, 1899–1900, **25**, 331.

10. HOOPER, C. W.; SMITH, H. P.; BELT, A. E., AND WHIPPLE, G. H.: Blood volume studies. I. Experimental control of a dye blood volume method. *Am. J. Physiol.*, 1920, **51**, 205.

11. KEITH, N. M.; ROWNTREE, L. G., AND GERAGHTY, J. T.: A method for the determination of plasma and blood volume. *Arch. Int. Med.*, 1915, **16**, 547.

12. LAMSON, P. D.; ABT, A. F.; OOSTHUISEN, C. A., AND ROSENTHAL, S. M.: The influence of the arterial blood supply to the liver on hemoglobin concentration in certain acute conditions. Part I. *J. Pharm. Exp. Therap.*, 1923, **21**, 401.

13. LAMSON, P. D., AND ROSENTHAL, S. M.: The inadequacy of our present blood volume methods. *Am. J. Physiol.*, 1923, **63**, 358.

14. LEE, F. W., AND WHIPPLE, G. H.: Blood volume studies. VI. Plasma volume as determined by hemoglobin injection. *Am. J. Physiol.*, 1921, **56**, 328.

15. LEHMANN, C. G., AND WEBER, ED.: Quoted from Lehmann. (*Physiol. Rev.*, 1921, **1**, 204.) *Lehrb. d. physiol. Chem.*, 1850, **2**, 259.

16. LINDHARD, J.: Dye method for determining the blood-volume test in vitro. *Am. J. Physiol.*, 1926, **76**, 497.

17. LINDHARD, J.: A dye method for determining the blood volume in man. *Am. J. Physiol.*, 1926, **77**, 669.

18. MCQUARRIE, I., AND DAVIS, N. C.: Blood volume studies. IV. Blood volume as determined by the change in refractivity of the serum non-protein fraction after injection of certain colloids into the circulation. *Am. J. Physiol.*, 1920, **51**, 257.

19. MEEK, W. J., AND GASSER, H. S.: Blood volume. A method for its determination with data for dogs, cats and rabbits. *Am. J. Physiol.*, 1918, **47**, 302.

20. MENDERSHAUSEN, A.: Blutmengenbestimmungen mit der Kongorotmethode. *Z. klin. Med.*, 1923, **97**, 468.

21. PLESCH, J.: Untersuchungen über die Physiologie und Pathologie der Blutmenge. *Z. klin. Med.*, 1922, **93**, 241.

22. SALVESEN, H. A.: The determination of blood volume by the carbon monoxide method. *J. Biol. Chem.*, 1919, **40**, 109.

23. SMITH, H. P.: Blood volume studies. II. Repeated determination of blood volume at short intervals by means of the dye method. *Am. J. Physiol.*, 1920, **51**, 221.

24. SMITH, H. P.: The fate of an intravenously injected dye (brilliant vital red) with special reference to its use in blood volume determination. *Johns Hopkins Hosp. Bull.*, 1925, **36**, 325.

25. SMITH, H. P.: Intravenous injections of fluid and repeated blood volume determinations. *Johns Hopkins Hosp. Bull.*, 1925, **37**, 177.

26. SMITH, H. P.; ARNOLD, H. R., AND WHIPPLE, G. H.: Blood volume studies. VII. Comparative values of Welcker, carbon monoxide and dye methods for blood volume determinations. Accurate estimation of the absolute blood volume. *Am. J. Physiol.*, 1921, **56**, 336.

27. Van Slyke, D. D., and Robscheit-Robbins, F. S.: The gasometric determination of small amounts of carbon monoxide in blood, and its application to blood volume studies. *J. Biol. Chem.*, 1927, **72**, 39.
28. Van Slyke, D. D., and Salvesen, H. A.: The determination of carbon monoxide in blood. *J. Biol. Chem.*, 1919, **40**, 103.
29. Welcker, H.: Bestimmung der Menge des Körperblutes und des Blutes der einzelnen Organe. *Prager Vierteljahrschrift für prakt. Heilkunde.:* 1854, **44**, 63.
Bestimmungen der Menge des Körperblutes und der Blutfärbekraft, sowie Bestimmungen von Zahl, Maass, Oberfläche und Volum des einzelnen Blutkörperchens bei Thieren und bei Menschen. *Zeitschr. f. rat. Medic.*, 1858, **4**, 145.
30. Whipple, G. H.: The hemoglobin of striated muscles. I. Variations due to age and exercise. *Am. J. Physiol.*, 1926, **76**, 693.

CHAPTER XXIII

Total Base

DISCUSSION

For many biochemical purposes, such as estimation of the total electrolyte content of biological solutions, or the measurement of the loss of fixed base during pathological conditions, a determination of the total fixed bases, Na + K + Ca + Mg, serves in place of separate estimations of the individual alkalies. Fiske (1) developed for urine a total base method in which the material was ignited with sulfuric acid, turning all the bases into sulfates. The amount of total base was ascertained by determining with the benzidine titration method (discussed on p. 890, in the sulfur chapter) the SO_4 content of the sulfate mixture. The method is accurate, relatively convenient, and avoids the possible errors involved in some of the quick micro methods for estimating sodium and potassium by complex precipitates of readily variable composition. Van Slyke, Wu and McLean (4) applied the principle of Fiske's method to the estimation of total base in blood after the proteins had been removed by trichloroacetic acid. They weighed the SO_4 as $BaSO_4$. Several investigators, including Cullen, Gamble, Loeb and Atchley, Stadie and Ross (2) and the writers have employed micro modifications of the blood method in which conditions were fitted to the benzidine titration, and by informal exchange of experiences have developed a fairly uniform practical procedure. The modifications described below have been developed in detail by Dr. Alma Hiller and Miss Carter Lee for routine use in the laboratories of the authors.

Van Slyke, Hiller, and Berthelsen have developed a quite different procedure for determining the SO_4 in the total base method. They shake the solution of sulfates with pulverized insoluble barium iodate. The reaction, $Ba(IO_3)_2 + Na_2SO_4 = BaSO_4 + 2\ NaIO_3$, occurs, SO_4 being precipitated and an equivalent of IO_3 going into solution. The dissolved IO_3 is determined gasometrically by measuring the N_2 gas evolved by reaction with hydrazine (see gasometric methods, p. 401). This method has an advantage in that it requires but a small amount of material, 1 cc. of serum sufficing for duplicate analyses. The dissolved IO_3 could also be determined by iodometric micro-titration.

Because of the extreme inequality of the distribution of sodium, potassium and total base between cells and serum, analyses of whole blood for these constit-

uents are of no value for clinical purposes. If knowledge of the distribution of these constituents between cells and serum is desired, one of two courses can be pursued. Either cells and serum can be analyzed separately: or less accurately cells and whole blood can be analyzed, cell volume can be determined simultaneously by means of the hematocrit (see p. 73) and cell content of base calculated from these data. *Precautions must be taken to prevent transfer of water across the cell membrane as the result of changes in the CO_2 and O_2 content of the blood* (see p. 1020–25 of Volume I).

The total base method is not applicable to feces. Presumably because of the frequently high calcium content, and failure to redissolve all the $CaSO_4$ formed, the results are likely to be low. Sometimes a urine with very high calcium content also gives a low result, but the error is infrequent.

TITRATION OF TOTAL BASE IN URINE AND SERUM BY BENZIDINE SULFATE METHOD. ADAPTATION OF FISKE'S (1) METHOD BY STADIE AND ROSS (2), HILLER AND LEE

Reagents

Sulfuric acid, 4.0 N.

Nitric acid, concentrated.

Hydrogen peroxide, 30 per cent (Merck's "superoxol").

Concentrated ammonia solution.

Dilute ammonia solution. One volume of concentrated ammonia plus 9 volumes of water.

Ferric ammonium sulfate. 3.18 grams $Fe\ (NH_4)\ (SO_4)_2 \cdot 12H_2O$ in 100 cc. of water.

Benzidine solution. Four grams para-benzidine in 45 cc. 1 N hydrochloric acid diluted to 250 cc. with distilled water. If a brown residue forms, the solution should be filtered through ash-free filter paper before it is used. This solution contains 175 milli-equivalents of benzidine hydrochloride and 5 milli-equivalents of free HCL per liter.

Acetone, 95 per cent.

0.1 per cent phenol red indicator solution.

Methyl orange, 0.1 per cent solution in 50 per cent methyl alcohol.

One per cent alcoholic phenolphthalein indicator solution.

Sodium hydroxide, 0.02 N. This is prepared by dilution of approximately carbonate-free 0.1 N solution with CO_2-free water. (For preparation of 0.1 N solution, see p. 29–30.)

Procedure

Preparation of material. The urine sample should contain an amount of chloride equivalent to 10 to 25 mg. of NaCl, and not more than 5 mg. of inorganic phosphorus. The volume of urine ordinarily taken is 1 to 2 cc.

Of serum a 1 cc. sample is taken.

The sample of either urine or serum is placed in a large Pyrex test tube (25 by 200 mm.). Two cubic centimeters of 4 N sulfuric acid, 1 cc. of concentrated nitric acid, and a glass bead are added. Heat a few minutes until a dark brown color appears, cool, add more nitric acid and heat again. Repeat this process two or three times, until the liquid is perfectly clear and all brown fumes have been driven off. The digestion can be greatly accelerated by adding to the cooled mixture, just after intense charring has disappeared and the fluid has assumed a yellow color, 5 drops of 30 per cent hydrogen peroxide (Merck's reagent "Superoxol").

Removal of the proteins of serum by precipitation with trichloroacetic acid would facilitate the ashing by greatly reducing the amount of organic matter to be burned. However, Van Slyke, Hiller, and Berthelsen (3) found that when an aliquot of the trichloroacetic acid filtrate of serum was analyzed the result was 154 m.-Eq. of base per liter, compared with 148 when the entire serum was ashed as above described. An investigation of known mixtures showed that the results by the ashing technique were the correct ones. Apparently when the proteins of serum are coagulated with trichloroacetic acid the coagulum contains less fixed base per cubic centimeter than the supernatant fluid, so that analysis of the supernatant liquid yields high results when they are estimated on the assumption that the fixed base cations are evenly distributed through coagulum and solution.

W. S. Hoffmann (The micro determination of fixed bases, calcium, and sulfates in urine: *J. Biol. Chem.*, 1931, 93, 787) avoids preliminary wet ashing of *urine* by applying Fiske's ferric acetate treatment for removal of phosphates to the complete urine. Hoffmann states that the basic ferric precipitate removes not only phosphates, but also all other interfering substances, including protein, lipoids, and any débris present, along with a large part of the urinary pigment. He carries out the precipitation as follows: 15 cc. of urine are measured into a Pyrex test tube marked for 25 cc. The urine is made faintly acid to methyl red by addition of either 10 per cent acetic acid or 10 per cent ammonia. Of a 10 per cent solution of ferric chloride in 0.2 N hydrochloric acid, 2 cc. are then slowly added, and are followed by 4 cc. of a 5 per cent solution of ammonium acetate. The solution is made up to 25 cc. and is carefully heated, with continual agitation, over a free flame until boiling just begins. The flame is then withdrawn. The voluminous precipitate which forms at the boiling point is at once filtered through a small ash-free filter paper. About 22 cc. of filtrate are obtained. The precipitate should have the brick-red color of basic ferric acetate. If it is brownish white, the quantity of ferric chloride added was insufficient to precipitate all the phosphate. This result occurs only with most concentrated urines. In this case only 10 cc. of urine are taken.

For total base determination 2 cc. of the filtrate, equivalent usually to 1.2 cc. of urine, are treated as described below under "Ignition of alkali sulfates."

Other portions of the filtrate can be used for determination of sulfates by the benzidine method and of calcium and magnesium.

Removal of phosphoric acid. After allowing the residue to cool, add 2 cc. of water and transfer the solution to a 25-cc. volumetric flask, washing with 4 additional 2 cc. portions of water. Add 1 drop of 0.1 per cent phenol red to the solution and render it just alkaline with concentrated ammonia solution. The indicator may conveniently be introduced with the last portion of wash water added to the digestion tube. In this manner it serves to show whether all the acid has been washed out of the tube. Then add by drops 4 N H_2SO_4 enough to make it just acid again. Add 1 cc. of ferric ammonium sulfate solution, and then dilute ammonium hydroxide till the full red alkaline color of the indicator develops. Dilute the mixture to the 25-cc. mark and filter off the ferric phosphate and hydroxide on an ash-free 5 to 6 cm. filter paper.

For removal of phosphate from urine, the 1 cc. of ferric ammonium sulfate solution will not in all cases suffice. One cc. of this solution is required for each miligram of inorganic phosphorus present. It is well to make a rough colorimetric determination of the phosphate in the urine before beginning the total base determination, and then take 1 cc. of the ferric solution for each mg. of P found.

Ignition of the alkali sulfates. Transfer 20 cc. of the filtrate to a silica or platinum dish and evaporate to dryness on the steam bath. The more completely the residue is dehydrated the less danger there will be of loss due to decrepitation and spattering in the subsequent ignition. It is well to let the dish stand over night on the hot steam bath. When the residue is as dry as possible, transfer it to a cold 2 or 3 step electric hot plate and cover it with a watch glass. Turn on the heat slowly. The dry material at first tends to flake off and crepitate. If it was thoroughly dried on the steam bath, flakes now thrown off drop back without loss. Gradually increase the heat until the material melts and sulfuric acid fumes appear. Then remove the cover glass and turn on full heat until the residue is dry. If silica dishes are used they are then heated fifteen minutes in the full flame of a triple Bunsen burner. If platinum dishes are used they are heated to a red heat for two minutes with a micro burner. Every part of the dish should be brought to a red heat 2 or 3 times. There is no danger of overheating under the conditions given; error is more likely to arise from failure to drive off free sulfuric acid completely.

Testing neutrality of residue. If any acid sulfate is left after the ignition, the results will be high. After the dry residue has cooled, it is transferred with five 2-cc. portions of water to a lipped 50-cc. Pyrex glass centrifuge tube. A drop of phenolphthalein is added and the solution is titrated to the neutral point with 0.02 N sodium hydroxide. If the ignition has been complete, so that all the H_2SO_4 has been driven off and the $BHSO_4$ changed to B_2SO_4, this preliminary titration will require no more alkali than the amount, about 0.05 cc. of 0.02 N NaOH, required to turn the 10 cc. of water alkaline to phenolphthalein. If more alkali is required in the preliminary titration, the difference is added to B in the calculation given below, in order to correct for excess SO_4 left in the form of acid sulfate after ignition. E.g., if the preliminary titration in an analysis requires 0.09 cc. of 0.02 N alkali, while to give the end-point with 10 cc. of water only 0.05 cc. is required, then 0.04 cc. is, for that particular analysis, added to the regular value of B.

Precipitation and filtration of benzidine sulfate. To the neutral solution in the tube are added 2 cc. of benzidine solution and 4 cc. of 95 per cent acetone. After the mixture has stood ten minutes it is filtered through a 5 or 6-cm. ash free filter fitted into a small funnel. The tube and precipitate are washed with 15 cc. of 95 per cent acetone, using four or five 1-cc. portions to wash the tube, the remainder to wash the precipitate on the filter paper. The filter paper is loosened from the funnel, allowed to rest a few minutes until some of the acetone has evaporated, and then transferred back to the tube in which the benzidine sulfate was precipitated.

Titration of the benzidine sulfate. To the tube containing the precipitate are added 10 cc. of water. The tube is then heated in a water bath until the odor of acetone disappears. One drop of phenolphthalein is now introduced and the solution is titrated while hot with 0.02 N sodium hydroxide to the first faint permanent pink, with care to observe that all particles of the benzidine sulfate precipitate have disappeared. If they have not, reheat and titrate until all particles have disappeared. Use a burette graduated into divisions of not more than 0.05 cc. so that readings can be estimated to 0.01 cc.

Calculation

$$\frac{25\,(A - B)}{\text{cc. urine sample}} = \text{milli-equivalents of base per liter of urine.}$$

$$25\,(A - B) = \text{milli-equivalents of base per liter of serum.}$$

A = cubic centimeters of 0.02 N NaOH used in titration of the benzidine sulfate.

B = cubic centimeters of 0.02 N NaOH used in the final titration in a blank analysis. In some analyses this value of B may require increase in the manner described above under "testing neutrality of residue."

The factor 25 is derived as follows. After precipitation of the phosphate 20/25 of the filtrate, equivalent to 0.8 cc. of serum, is used for analysis. The cubic centimeters of 0.02 N NaOH are therefore multiplied by $\frac{1000}{0.8}$ to give cubic centimeters of 0.02 N base per liter of serum and by 0.02 to give the cubic centimeters of 1 N or milli-equivalents, per liter. Multiplying together the factors $\frac{1000}{0.8}$ and 0.02 gives 25 as the combined factor. Similarly, the base titrated in the urine analysis represents 0.8 of the sample.

Blank. Run through the procedure using all reagents and processes described, omitting the serum.

Alternative procedure of Stadie and Ross (2), for titration of the precipitated benzidine sulfate by difference

In this procedure the precipitate itself is not titrated, but an aliquot of the filtrate. The decrease in acidity caused by precipitating part of the SO_4 with benzidine serves as a measure of the SO_4 precipitated. This procedure has a marked advantage in time saved, because it avoids both the washing of the precipitate and the necessity for continued heating during the titration, required in the method above described, to redissolve the benzidine sulfate. It has the disadvantage that it is a titration by difference, in which the sources of possible error are multiplied by the involvement of the quantitative measurement of the volume of benzidine solition used, the volume of solution in which the precipitate is formed, and the volume of the filtrate taken for titration. In such a titration, accuracy of 1 part per 1000 in the individual measurements is necessary to keep the error of the final result within 1 part per 100 (see "Accuracy in volumetric analysis" p. 34–36).

Up to the precipitation of benzidine sulfate, the analysis is the same as the above. From that point the following technique is followed.

Precipitation of benzidine sulfate. **The redissolved sulfates are transferred to a 20 cc. volumetric flask instead of a test tube, and the preliminary titration is performed in the flask. The solution is then brought by addition of water (no acetone) to about 15 cc., and 2.00 cc.**

of benzidine solution, accurately measured from a pipette, are added. The volume is then made up to 20 cc.

Titration of filtrates. The solution is filtered through a small dry quantitative filter paper into a small dry Erlenmeyer flask. Of the filtrate 15 cc. are pipetted into another flask and titrated with 0.02 N sodium hydroxide, with a drop of phenolphthalein solution as indicator.

A ***blank analysis*** is done in the same way on the reagents.

Calculation

$$\frac{33.3\ (B - A)}{\text{cc. urine sample}} = \text{milli-equivalents of base per liter of urine.}$$

$$33.3\ (B - A) = \text{milli-equivalents of base per liter of serum.}$$

B = cubic centimeters of 0.02 N NaOH used in titration of the blank filtrate.

A = cubic centimeters of 0.02 N NaOH used in titration of the filtrate in the urine or serum analysis.

If the preliminary titration requires more alkali than necessary to give the end-point with water, the extra cc. of 0.02 N NaOH are added to A.

PREPARATION OF MATERIAL FOR DETERMINATION OF TOTAL BASE, SODIUM, AND POTASSIUM IN A SINGLE SAMPLE OF URINE

In studies of acid and base balance it is sometimes of interest to determine in the urine the total base and sodium and potassium without attention to the proportions of calcium and magnesium, which are of less importance from the standpoint of water and acid-base economy. For this purpose Miss Evelyn B. Man, in the laboratory of one of the authors, has developed a combination of methods which consists of the conversion of all the bases to sulfate by the procedure employed in the determination of total base, the analysis of aliquots for total base by the technique above described, and sodium and potassium by micro methods described in the next chapter. For potassium Shohl and Bennett's titration method was used (see p. 741), for sodium the pyroantimonate titration (see p. 737), now preferably replaced by the uranyl zinc acetate gravimetric method (p. 732).

Procedure

Enough material is taken to contain 4.5 to 7.5 mg. of sodium and 1.5 to 6.0 mg. of potassium.

Digestion and conversion to sulfate. The material is digested

over a micro burner in large Pyrex tubes with 2 cc. of 4 N sulfuric acid, about 10 drops of concentrated nitric acid and 5 to 10 drops of superoxol, until the liquid is clear and colorless and fumes of nitric oxide have ceased to come off.

The residue is then transferred to a 25-cc. volumetric flask with five 2-cc. portions of water. Dilute ammonia solution is added until the solution is alkaline to phenol red and then 4 N sulfuric acid until it is acid again. Enough ferric ammonium sulfate solution is then introduced to precipitate all the phosphates (1 cc. for each milligram of P). The solution is made alkaline with ammonium hydroxide, made up to 25 cc., mixed and filtered through ash-free filter paper.

Twenty cubic centimeters of the filtrate are transferred to a platinum or silica dish and evaporated to dryness on the steam-bath. The dish is then placed on an electric hot plate the heat of which is gradually increased as the drying process advances. The dried material is finally ashed over a micro burner, as described above for the determination of total base.

Preparation of ash for different determinations. The ash is transferred to a 25-cc. volumetic flask, washing with several portions of water, and diluted to volume. For the various determinations the following aliquots are used:

Total base, 5 cc.
Sodium, 15 cc. (2 to 3.75 mg. Na).
Potassium, 4 cc. (0.2 to 0.8 mg. K).

For determination of total base the 5-cc. aliquot, in a lipped 50-cc. Pyrex centrifuge tube, is diluted with 5 cc. of water and 2 cc. of benzidine solution are added, followed after two minutes by 4 cc. of 95 per cent acetone. The micro methods for sodium and potassium are described in the next chapter.

GASOMETRIC MICRO METHOD FOR TOTAL BASE (3)

This method, by which duplicate analyses can be done with a single 1 cc. sample of serum, is described on page 401 of the chapter on gasometric methods.

BIBLIOGRAPHY

1. Fiske, C. H.: A method for the estimation of total base in urine. *J. Biol. Chem.*, 1922, **51**, 55.
2. Stadie, W. C., and Ross, E. C.: A micro method for the determination of base in blood and serum and other biological materials. *J. Biol. Chem.*, 1925, **65**, 735.

3. Van Slyke, D. D., Hiller, A., and Berthelsen, K.: A gasometric micro method for determination of iodates and sulfates, and its application to the estimation of total base in blood serum. *J. Biol. Chem.*, 1927, **74**, 659.
4. Van Slyke, D. D., Wu, H., and McLean, F. C.: Studies of gas and electrolyte equilibria in blood. V. Factors controlling the electrolyte and water distribution in the blood. *J. Biol. Chem.*, 1923, **56**, 765.

CHAPTER XXIV

SODIUM AND POTASSIUM

DISCUSSION

In this chapter it is convenient to deviate from the arrangement of describing the methods, first for urine, then for feces, and finally for blood. Instead we shall describe in turn each method used, and with the description the applications of the method to the different materials.

The classical macro gravimetric methods first described are the most exact and serve well for urine and feces. They take time, however, and of serum, unless a micro balance is used, they require at least 5 cc. for an analysis, an amount frequently not available. Consequently for micro analyses recourse has been had to methods which can be carried through more rapidly and with small amounts, and which still yield results sufficiently exact to be significant for many clinical and physiological studies.

The gravimetric zinc uranyl acetate method of Kolthoff for sodium approaches in accuracy the classical methods, and permits a micro analysis with the technique of macro gravimetric determinations, because of the great weight of the precipitate. When Na is much less than K, the uranyl method is, in fact, more exact for Na than is the classical method. In the latter, Na and K are determined together as chlorides or sulfates. K is then determined as chloroplatinate or perchlorate, and Na is calculated by subtracting K from Na + K. Consequently when Na is much less than K, all errors are heaped on the small amount of Na. The direct uranyl method for Na suffers from no such cumulative error.

For potassium, micro titration and colorimetric methods based on the platinic chloride precipitate have been devised by Shohl and Bennett which are exact within the limits of technical error of the methods of measurement.

Other micro methods for sodium and potassium which are intrinsically less exact than those mentioned above will nevertheless be described for specific reasons. The pyroantimonate micro sodium method is outlined because it offers the only micro titration method for sodium. It is capable of giving good results, but is susceptible to error from slight changes in technique, and the potassium pyroantimonate used as reagent obtained from dealers has often been found unsatisfactory for reasons which have not been defined. The cobaltinitrite method for potassium is based on the

precipitation of potassium sodium cobaltinitrite in which the potassium content varies with the conditions of precipitation. For this reason the cobaltinitrite method is susceptible to errors from which the platinochloride methods are free. The cobaltinitrite precipitate, however, lends itself with especial convenience to micro determinations, and by means of it most of the present data on blood potassium content in the literature have been obtained.

GRAVIMETRIC DETERMINATION OF SODIUM AND POTASSIUM AS THE COMBINED SULFATES AND POTASSIUM CHLOROPLATINATE

In the classical methods of analytical chemistry the heavy metals and alkaline earths are first removed, then potassium and sodium are weighed together as sulfates or chlorides. For this weighing the sulfates have an advantage over the chlorides in that the former are not volatile at the ordinary heat of ignition, while chlorides are easily lost by volatilization if at all overheated. From the redissolved chlorides the potassium can be precipitated and weighed either as the chloroplatinate, K_2PtCl_6, or as the perchlorate, $KClO_4$. With the redissolved sulfates only the chloroplatinate method for potassium can be used, since SO_4 prevents combination of potassium with ClO_4. After the potassium has been determined as either chloroplatinate or perchlorate, the sodium is determined by subtracting the K from the Na + K already measured by weighing the combined chlorides or sulfates.

The method here detailed is the classical Lindo-Gladding procedure which is official with the Association of Official Agricultural Chemists (19). In the form described below it has been extensively used by MacKay and Butler in the laboratory of one of the authors for analyses of urine, feces, and blood. Sodium and potassium are weighed together as sulfates, and potassium is determined as the chloroplatinate.

Reagents

Hydrogen peroxide, 30 per cent ("superoxol" Merck's blue label).
Trichloroacetic acid, 25 per cent.
Concentrated nitric acid (specific gravity 1.42).
Methyl red, a saturated solution in 95 per cent alcohol.
Concentrated ammonia solution (specific gravity 0.90).
Barium chloride, 10 per cent solution.
Ammonium carbonate, saturated solution.
Concentrated sulfuric acid (specific gravity 1.84).
Chloroplatinic acid, 10 per cent solution.

Concentrated hydrochloric acid (specific gravity 1.178 to 1.185).

Ethyl alcohol, 80 per cent (specific gravity 0.86) made from alcohol redistilled over lime.

Ammonium chloride, 20 per cent, saturated with potassium chloroplatinate by shaking the solution at intervals for a few days with a small amount of the finely pulverized salt.

PROCEDURE WITH URINE. MACKAY AND BUTLER (UNPUBLISHED)

Preparation of urine sample. It is convenient, especially if other analyses are contemplated, to dilute the urine specimen to an even volume of 2 or 3 liters before taking samples for analysis. The sample for a single analysis should contain from 1.5 to 5 milli-equivalents of sodium plus potassium and preferably not less than 0.5 milli-equivalents of either (5 milli-equivalents = 115 mg. Na or 195 mg. K). The amount needed varies from 10 per cent of the twenty-four-hour specimen if the subject has received a salt-poor diet to 1 per cent if he has received a high salt diet. Twice the required amount may be prepared and duplicate aliquots taken after the first precipitation. If the urine contains protein, 30 cc. of 25 per cent trichloroacetic acid are added to 120 cc. of the urine, the solutions are mixed and the mixture is filtered through ash-free filter paper. To a suitable volume of urine or its protein-free filtrate, in a 50-cc. wide mouthed Erlenmeyer flask, are added 10 cc. of concentrated nitric acid and 15 cc. of 30 per cent hydrogen peroxide. The mixture is evaporated to dryness on the water bath; the residue is dissolved in 30 to 40 cc. of hot water and rinsed, with about 150 cc. of hot water, into a 250-cc. (500-cc. if a double sample was taken) volumetric flask, preferably of Pyrex glass.

Removal of calcium, magnesium and phosphates. From this point on the procedure for urine, feces and blood is identical. Three drops of methyl red are added to the suspension in the volumetric flask, which is then made alkaline with concentrated ammonia solution. An additional 10 cc. of ammonia is then added. An excess of 10 per cent barium chloride is introduced to remove the phosphate not already precipitated and all of the sulfate. The addition of an excess of saturated ammonium carbonate then removes the barium, any calcium which remains, and all the magnesium. If possible this entire precipitation is carried out while the solution is still warm. After the addition of the ammonim carbonate the mixture is well shaken and allowed to stand at least three hours, preferably over night. It is then made up to volume and filtered through an ash-free folded filter (Schleicher

and Schüll, 589, Blue Ribbon, 15 cm. is suitable). Then 200 cc. of the filtrate are evaporated to dryness in a casserole on the steam bath. This drives off most of the ammonia and ammonium carbonate which, if neutralized, would yield a large amount of troublesome, creeping ammonium salts.

Weighing the combined sulfates. In many specimens an appreciable amount of barium, and in some stool samples considerable calcium, may escape precipitation as carbonate and appear when the filtrate is concentrated. Small amounts of barium and calcium which come through to this point are removed in the following manner. To the residue in the casserole, dissolved in 10 cc. of water with the aid of heat, is added 1 drop of concentrated sulfuric acid. The solution is made alkaline with a few drops of concentrated ammonia solution and 0.5 cc. of saturated ammonium carbonate is added. Then the cool solution is filtered through an analytical filter (Schleicher and Schüll, 589, Blue Ribbon, 9 cm.), in a log-stemmed funnel, into a weighed 75-cc. platinum dish, the edge of which is greased with vaseline. Casserole and filter are well washed with small portions of lukewarm water until the filtrate amounts to about 60 cc. Five-tenths cubic centimeter of concentrated sulfuric acid is now added and the filtrate is evaporated as nearly to dryness as possible on the steam bath. The platinum dish is then transferred to a cold electric hot plate and the temperature is gradually raised. When the acid and ammonium salts have been driven off as far as possible (this usually requires several hours), ashing is completed by heating the dish carefully, first over an ordinary Bunsen burner and then over a triple burner, or in a slowly heated muffle furnace. When a bright red heat is obtained it should be maintained for at least 30 minutes. In a pyrometer controlled muffle furnace the maximum temperature should be 600 to 700°. The ignited sulfates are cooled in a disiccator and weighed.

Precipitation of potassium chloroplatinate. The combined sulfates are dissolved in a few cubic centimeters of hot water and washed from the platinum dish into a small porcelain evaporating dish with about 10 to 15 cc. of water for each 0.1 gram of sulfates. Several drops of concentrated hydrochloric acid and 2.5 cc. of 10 per cent chloroplatinic acid are added for each 0.1 gram of sulfates. The mixture is evaporated to dryness on the steam bath. Great care must be exercised to prevent access of ammonia, which forms an insoluble chloroplatinate. Ten to 20 cc. of 80 per cent alcohol (from alcohol redistilled over lime to eliminate aldehydes, which form insoluble compounds with chloro-

platinic acid) are added to the residue and allowed to remain on it for thirty minutes to remove any unprecipitated chloroplatinic acid. The mixture is then washed into a weighed Gooch or porous glass crucible (15-cc. Jena glass crucible with a porous glass filtering disc of medium porosity, 10 G-3) and numerous small portions of 80 per cent alcohol are used to wash particles out of the porcelain dish and to wash the precipitate free of chloroplatinic acid. Suction is used and alcohol washing is continued until the filtrate is clear and colorless. The potassium chloroplatinate is then washed free of sodium sulfate with numerous 5-cc. portions of 20 per cent ammonium chloride saturated with potassium chloroplatinate. The adherent ammonium chloride is removed by further washing with small volumes of 80 per cent alcohol. All of the filtrates are saved for recovery of the platinum. The precipitate is dried for four hours at 110° and then weighed.[1]

PROCEDURE WITH FECES. MACKAY AND BUTLER (UNPUBLISHED)

Preparation of sample. The stool specimens are washed into a 1-liter Erlenmeyer flask of Pyrex glass. For each twenty-four-hour stool about 300 cc. of water are added and, while the mixture is stirred at a high rate of speed with a mechanical stirrer, 250 cc. of concentrated sulfuric acid are slowly added. (Stool collected in sulfuric acid for nitrogen determinations (see p. 78) can be used). Enough water is added to bring the volume almost to a liter, the solution is cooled, transferred to a liter volumetric flask and diluted to volume, a few drops of caprylic acid serving to destroy the foam. For stool specimens of more than twenty-four hours proportionately larger volumes are used throughout. The stools may be made up to a given weight, if preferred. Aliquots of the resulting suspension are used for analysis. The stool container is well shaken before a sample is withdrawn. If the subject has received a diet containing no added salt, 100 cc. (or 100 grams) is a suitable sample; if the diet contains the usual amount of added salt 75 cc. (or 75 grams) is sufficient for a single analysis. This sample is transferred to a 500-cc. Kjeldahl flask with several glass beads and

[1] The platinum is regained from the mother liquors and washings and from the redissolved precipitates by precipitation with ammonia. The washed precipitate is ignited to platinum black, which can be redissolved in aqua regia to form fresh platinic chloride solution. Or, by letting the mother liquors, etc., stand with an excess of granulated zinc the platinum can be reduced directly to the metallic form. The solution of platinic chloride made with aqua regia is heated with excess hydrochloric acid to decompose all the nitric acid present.

boiled in a Kjeldahl digestion rack until acid fumes are given off. The flask is then cooled and 5 cc. of 30 per cent hydrogen peroxide are cautiously added. After effervescence has ceased boiling is resumed and continued for several minutes. The mixture is then cooled again another portion of hydrogen peroxide is added and boiling is repeated. In all, treatment with hydrogen peroxide is repeated from 3 to 6 times until a clear, colorless or light yellow solution is obtained. This is cooled and rinsed into a small porcelain casserole with a little water. The acid is removed by evaporation on a very hot electric plate. The last remnant is driven off by heating the residue in a muffle furnace or over a triple Bunsen burner. The residue in the casserole is dissolved with the smallest possible volume of concentrated hydrochloric acid and washed into a 250-cc. (500-cc. if a double sample was taken) volumetric flask of Pyrex glass with enough hot water to bring the volume to about 175 cc.

The procedure from this point on is identical with that described above, beginning with the heading "Removal of calcium, magnesium, and phosphates" for the analysis of urine.

PROCEDURE WITH BLOOD AND SERUM. MACKAY AND BUTLER (UNPUBLISHED)

The sample should represent if possible 10 cc. of blood or serum, although by careful work results can be obtained with 5 cc. The whole blood or serum may be ashed by the Stolte method (p. 70) or by the procedure described above for feces. The ash is redissolved in warm water plus a few drops of concentrated hydrochloric acid and analyzed as described above for urine. The weighings are done in small crucibles.

Calculation

Grams of K in sample $= 0.1608\ W_2$

Grams of Na in sample $= 0.3238\ (W_1 - 0.3583\ W_2)$

Milli-equivalents of K in sample $= 4.112\ W_2$

Milli-equivalents of Na in sample $= 14.08\ (W_1 - 0.3583\ W_2)$

Milli-equivalents of K per liter of urine or blood $= \dfrac{4112\ W_2}{V}$

Milli-equivalents of Na per liter of urine or blood $\dfrac{14080\ (W_1 - 0.3583\ W_2)}{V}$

W_1 is the weight in grams of the combined Na_2SO_4 and K_2SO_4 from the unknown minus their weight obtained in a blank determination.

W_2 is the weight in grams of the K_2PtCl_6 from the unknown minus the weight obtained in a blank determination.

V is the volume in cubic centimeters of urine or blood represented in the weighed sulfates.

GRAVIMETRIC DETERMINATION OF SODIUM IN URINE. BUTLER AND TUTHILL'S (5A) APPLICATION OF THE METHOD OF BARBER AND KOLTHOFF (3)

Kolthoff (12) in 1927 proposed, as a means of detecting sodium, its precipitation as uranyl zinc sodium acetate. More recently, with Barber (3) he has shown that the same precipitation can be applied to the gravimetric quantitative determination of sodium. It lends itself to micro analysis because of the large weight of the uranyl zinc sodium acetate molecule The sodium is weighed as $(UO_2)_3\ ZnNa(CH_3COO)_9 \cdot 6H_2O$; the precipitate therefore weighs 67 times as much as the sodium in it. The hydrated salt is apparently quite stable and constant in composition. It is slightly soluble in water and even in alcohol; but error from solubility is obviated by using as a precipitating reagent uranyl zinc acetate solution saturated with the sodium salt, and by washing the precipitate with 95 per cent alcohol also saturated with the salt.

If the solution in which the precipitate is formed contains more than 50 mg. of potassium per cubic centimeter some of the potassium will be precipitated and results for sodium will be too high. However, in biological material such preponderant amounts of potassium seldom occur.

Preliminary removal of phosphates is necessary, because phosphate is precipitated as uranyl phosphate. Ashing of urine is not necessary, but proteins if present are removed by precipitation.

Powdered $Ca(OH)_2$ is used as the means of precipitating phosphate. Butler and Tuthill found it to be more convenient than the magnesia mixture previously used. Use of calcium hydroxide simplifies the reagents required for the procedure. Its addition clarifies cloudy urines and produces no volume change, thus eliminating the necessity of diluting to known volume and the taking of aliquots, or the quantitative washing of the precipitate. For urines containing protein a solid precipitating reagent, $HgCl_2$, is also used, again avoiding volume changes.

Reagents

1. *Uranium zinc acetate reagent.*

Solution A:

80 grams Na-free uranium acetate, $UO_2(C_2H_3O_2)_2 \cdot 2H_2O$.
48 grams (or 46 cc.) 30 per cent acetic acid (per cent by volume).
Add water to make 520 grams.

Solution B:

220 grams zinc acetate, $Zn(C_2H_3O_2)_2 \cdot 2H_2O$.
24 grams (or 23 cc.) of 30 per cent acetic acid.
Add water to make 520 grams.

Cover and warm both solutions on steam bath with occasional stirring until solution is complete. Mix while hot and let stand twenty-four hours before using. If no yellow precipitate appears add 0.2 gram of precipitated uranyl zinc sodium acetate in order to saturate with this triple salt. To assure saturation at temperature of analysis, shake occasionally, and filter as much solution as is needed immediately before using.

2. *Phenolphthalein,* 1 per cent alcoholic solution.
3. *Powdered* $HgCl_2$.
4. *Powdered* $Ca(OH)_2$.
5. *Saturated solution of ammonium perchlorate,* NH_4ClO_4.
6. *Ninety-five per cent alcohol saturated with the uranium sodium zinc acetate precipitate.*

A. Procedure for urines of ordinary Na content

Measure out roughly into a small flask 6 cc. of urine. Add 1 drop of phenolphthalein and 0.2 gram powdered calcium hydroxide. (If the urine contains protein take 10 cc. instead of 6, and add 0.05 gram $HgCl_2$. Then add the phthalein and calcium hydroxide.) Shake and let stand thirty minutes with occasional shaking. The solution should turn pink. Filter through a fine filter paper and collect the filtrate in a test tube. (If the urine contains protein, test the filtrate for protein. If protein is present, add more $HgCl_2$ and refilter.) Stopper the test tube in order to prevent precipitation of $CaCO_3$ by atmospheric CO_2.

Fit a solid rubber stopper from below into the bottom of a 30-cc. Jena glass, porous bottomed, filter crucible[2] that has been dried and weighed.[3] The filter now stands on the rubber stopper. The stopper prevents the liquid from going through the filter even though there is some air space between stopper and porous glass. Pipette approximately 20 cc. of freshly filtered reagent to the filter. (The reagent should be shaken frequently preceding use to insure saturation.) Pipette 2 cc. of urine filtrate directly into the reagent solution in filter.[4] If the pipette is

[2] Jena glass filter crucibles, size No. 2, capacity 30 cc., porosity 1 G4, may be obtained from the Empire Laboratory Supply Company, New York.

[3] Clean and dry with alcohol and ether, applying suction, and let stand one-half hour in desiccator; then weigh.

[4] When urines are unusually low in Na see modified procedures under (B) and (C).

graduated for blow-out delivery and one makes the delivery by blowing instead of warming by hand, a precipitation of $CaCO_3$ may occur. Aside from dirtying the pipette, this does not matter as this precipitate redissolves immediately. Stir with small glass rod till a precipitate appears, and several minutes thereafter. Stirring is particularly important where there is a small amount of Na present. Under such conditions insufficient stirring will give results from 2 to 5 per cent too low. Withdraw stirring rod, rinsing it with 3 to 5 cc. of reagent as it is withdrawn. Cover the filter with a watch glass and let it stand at approximately constant room temperature for one hour. Remove the rubber stopper, place the filter in a suction flask, and apply suction. After the reagent has been filtered off, wash the precipitate with five 2-cc. portions of 95 per cent alcohol saturated with the triple salt. The alcohol reagent should be filtered before use. Care should be taken to wash down the sides of the filter. Delaying the washing makes this more difficult. Finally wash with two 5-cc. portions of ether. Continue suction until the precipitate is thoroughly dry, place in desiccator over calcium chloride and weigh after one-half hour.

Run a blank on an equal volume of distilled water and reagents.

The ratio of volume of urine to volume of reagent must not be greater than 1 to 10. As the safe capacity of the filter is 25 cc. the largest urine filtrate that can be pipetted directly to the filter is 2.5 cc. Twenty-five cubic centimeters of the reagent will precipitate 20 mg. of Na. The likelihood of urines exceeding 20 mg. per 2 cc. is very slight. But because of the difficulty of washing large precipitates, it is better not to exceed a precipitate of about 700 mg. Since 1 mg. of Na gives a precipitate of 66.9 mg., the sample of urine taken for analysis should preferably not contain more than 10 mg. of sodium nor less than 1 mg.

Calculation

$$\text{Grams of Na in sample} = 0.01495\,(\text{grams of precipitate} - \text{grams of blank})$$

$$\text{Grams of Na per liter of urine} = \frac{14.95\,(\text{grams of precipitate} - \text{grams of blank})}{V}$$

$$\text{Milli-equivalents of Na per liter of urine} = \frac{650\,(\text{grams of precipitate} - \text{grams of blank})}{V}$$

V = cubic centimeters of urine in sample.

B. Procedure for urines low in sodium

Urines produced on low salt diets or as the result of certain types of diuresis may contain less than 0.5 mg. of Na per cc. When a urine con-

tains between 0.5 and 0.1 mg. of Na per cubic centimeter, a volume of urine filtrate containing between 1 and 3 mg. of Na, but not exceeding 10 cc., is pipetted into a small evaporating dish or beaker. One drop at a time of concentrated HCl is added until the urine turns acid. Acidification lessens the formation of precipitate during evaporation. The sample is evaporated down to approximately 2 cc. If it is evaporated down to dryness, 2 cc. of water are added. It is then transferred quantitatively to the reagent in the weighed filter as described for Procedure *A*, except that approximately 15 cc. of reagent is placed in the filter instead of 20 cc. The beaker is rinsed with 0.5 cc. of water and then with two 3-cc. portions of reagent. The solution in the filter is stirred the proper length of time and the stirring rod rinsed during removal with 5 cc. of reagent. The procedure is then continued as above outlined. Should the evaporation continue to dryness or a precipitate crystallize out, the transfer to reagent in filter is made as usual, with washing of all the precipitate to the reagent. Here the volume and acidity are such as to dissolve this residue. The stirring is continued a little longer in order to assure its complete solution.

When the volume of urine used necessitates this concentration procedure and a slimy residue remains after evaporation, one may add 5 cc. of concentrated HNO_3 plus 2 cc. of superoxol, cover, destroy organic matter by boiling, and then evaporate to dryness. This will facilitate the transfer but Butler and Tuthill state that it does not seem necessary, as they have incurred no demonstrable error in transferring such residues directly to the reagent in the glass filters.

Calculation as in Procedure A.

C. Procedure for urines of minimal sodium content

A very low sodium diet may in man cause excretion of urine so low in sodium that 40-cc. samples of urine are required in order to obtain accurately weighable precipitates. Such samples evaporated to 2.5 cc. for transfer to reagent have usually exceeded the permissible potassium concentration. To remove potassium from these samples, the following procedure serves, in which most of the potassium is precipitated as perchlorate.

Concentrate 50 cc. of urine and ash by the wet nitric acid plus superoxol method described under *B*. (The Stolte method of ashing has been found for this purpose unsatisfactory.) Extract the residue with warm water. Without filtering, transfer quantitatively to a 25-cc. volumetric flask and make up to volume. Mix well and then pour most of

the solution into a small Erlenmeyer flask. Add about 0.4 gram of solid $Ca(OH)_2$. Let this stand for about one hour with occasional shaking. Filter and test for PO_4 the first portion of filtrate. (Occasionally one may find phosphate. In this case the treatment with $Ca(OH)_2$ must be continued longer.) Of the filtrate transfer 20 cc. to a small beaker and evaporate this on a steam bath to dryness. Add 5 cc. of hot water and 1 drop of concentrated HCl; shake for a few minutes and then add 3 cc. of saturated ammonium perchlorate solution. Cool and let stand with occasional shaking for one-half hour. Filter into a beaker and wash quantitatively with 95 per cent alcohol. Evaporate the filtrate to dryness and transfer the entire contents to the uranium zinc acetate reagent in a glass filter as outlined previously, using 2 cc. of water for solution and transfer and 0.5 cc. for the first washing of the beaker. As the undissolved residue is fairly large, it is wise to use several 3-cc. portions of ***reagent*** to complete the transfer and rinsing of beaker. In this instance stirring must be continued longer in order to assure complete solution of the residue. Stir the solution, then let the rod remain in the solution in the filter for about ten minutes, then stir again, and then remove the rod with appropriate rinsing. The washing and weighing are carried out as usual. The sample analysed represents 40 cc. of urine.

Calculation as in Procedure A.

PROCEDURE FOR STOOLS

The material is prepared for analysis by evaporating to dryness, powdering, dry ashing, and extracting with dilute HCl. The actual determination of Na is done exactly as for urines except that particular attention should be paid to the complete precipitation of all the PO_4.

Calculation

Grams of Na in sample = 0.01495 (grams of precipitate − grams of blank)

PROCEDURE FOR SERUM

Pipette 1 cc. of serum to a thick-walled Pyrex test tube (200 by 25 mm.). Add a small crystal of quartz, 1 cc. of 4 N H_2SO_4 and 0.5 cc. concentrated HNO_3. Digest as one would in the usual micro Kjeldahl method. When charring appears remove the flame and add carefully down the side of the tube a few drops of superoxol or concentrated HNO_3. Digest again. If the solution does not clear, repeat the addi-

tion of H_2O_2 or HNO_3 and heating. After the solution has cleared, continue heating for a few minutes. Cool and add 4 to 5 drops of water, then pour into approximately 15 cc. of freshly filtered reagent in a glass filter, weighed and fitted into a stopper as mentioned in the procedure for urine. Rinse the contents of test tube to filter quantitatively with three 0.5-cc. portions of water, and finally two 3-cc. portions of reagent. Stir the solution in the filter till a precipitate appears and for several minutes thereafter. Withdraw the stirring rod, rinsing it with 3 cc. of reagent as it is withdrawn. Proceed with the determination as in the urine method.

Calculation

Milligrams of Na in sample = 0.01495 (milligrams of precipitate − milligrams of blank)

Milligrams of Na per 100 cc. of serum = 1.495 (milligrams of precipitate − milligrams of blank)

Milli-equivalents of Na per liter of serum = 0.65 (milligrams of precipitate − milligrams of blank)

The values for milligrams per 100 cc. and for milli-equivalents are calculated on the assumption that the serum sample is 1 cc.

TITRIMETRIC MICRO DETERMINATION OF SODIUM BY THE PYROANTIMONATE METHOD. KRAMER AND GITTLEMAN'S (14)

PROCEDURE MODIFIED BY EISENMANN (29)

Sodium in alcoholic solution is precipitated as sodium pyroantimonate $Na_2H_2Sb_2O_7 \cdot nH_2O$. The antimony of the precipitate is then determined by iodimetric titration. The pentavalent Sb is reduced by KI to trivalent, and the iodine liberated is titrated with thiosulfate.

$$Sb^{v} + 2\ KI \rightarrow Sb^{iii} + I_2.$$

Kramer and Tisdall (16) had earlier proposed gravimetric estimation of the pyroantimonate. In the application of the method most investigators encountered difficulties. These seemed to depend on several factors: the tendency for the finely crystalline precipitate to pass through filters, the inconstant amount of water of crystallization, and the inclusion with the precipitate of other substances which are insoluble in alkaline media. For these reasons iodimetric titration of the precipitate was proposed independently by Bálint (2) and by Kramer and Gittleman (14). Bálint also made a detailed study of the optimum conditions for quantitative precipitation of sodium pyroantimonate in analysis of serum. He concluded that sodium could be precipitated quantitatively from serum only if the serum

was first ashed, a conclusion in which he has been supported by Eisenman (29). Rourke (20) finds that ashing is unnecessary if the alcohol is added to serum at a temperature of only 10°C., thus avoiding precipitation of proteins.

Reagents

Hydrochloric acid, 0.1 N.

Potassium hydroxide, 10 per cent, free from sodium.

Potassium pyroantimonate. Briggs (6) first called attention to the variability of samples of pyroantimonate purchased on the market. This was further emphasized by Eisenman (29). Both antimony content and alkalinity of the salt vary greatly and such variations have a great influence upon the quality of the pyroantimonate reagents. Kahlbaum's reagent, pure $K_2H_2Sb_2O_7$, is to be used if possible. If other preparations are used, they must be tested for antimony content and reaction. The reagent as finally prepared should contain, in 10 cc., not more than 70 mg. of antimony and its pH should be about 9. It should contain no sodium and should form no precipitate when alcohol is added to it in the proportions used for a determination. The following directions for its preparation are those given by Kramer and Gittleman.

To 500 cc. of boiling water in a Pyrex glass flask, add approximately 10 grams of Kahlbaum's potassium pyroantimonate. The boiling is continued from three to five minutes. Then the mixture is rapidly cooled under running water. To the cold solution are added 15 cc. of 10 per cent sodium-free potassium hydroxide. The reagent is filtered through ash-free filter paper into a paraffined bottle. Some of the undissolved potassium pyroantimonate may pass through the paper. However, this will settle to the bottom after twenty-four hours, leaving a clear supernatant fluid which is quite satisfactory. The solution keeps well at room temperature; but must be discarded if it is not entirely clear. The reagent should give a perfectly clear solution when mixed with one-fifth its volume of 95 per cent alcohol.

Alcohol, 95 per cent, redistilled.

Alcohol, 30 per cent; 30 cc. of redistilled 95 per cent alcohol diluted to 95 cc. with water.

Hydrochloric acid, 10 N.

Potassium iodide, 2 per cent solution.

Sodium thiosulfate, 0.1 N, standardized against potassium biiodate or iodate (see p. 33).

Starch indicator solution, prepared as described on page 34.

Preparation of material

Serum is ashed by the Stolte process (p. 70) or by the wet ashing method with sulfuric and nitric acids and hydrogen peroxide described in the total base chapter (see p. 719). The ash is redissolved in a volume of 0.1 N hydrochloric acid equal to the volume of serum ashed.

Whole blood may be similarly ashed. The ash is redissolved in a volume of 0.1 N hydrochloric acid equal to one-half the volume of the blood from which the ash was obtained. It is necessary to take more blood than serum because the sodium content of the cells is low.

Urine is ashed by the Stolte process. The ash is dissolved in the smallest possible volume of 0.5 N hydrochloric acid and is then diluted with about 4 times as much water, so that the concentration of HCl is brought to approximately 0.1 N. It is then diluted with 0.1 N hydrochloric acid to such a volume that 1 cc. of the ash solution contains from 2.00 to 3.75 mg. of Na.

Feces. Dried, powdered feces may also be ashed by the Stolte method (see p. 70), and the ash dissolved in hydrochloric acid.

Precipitation and titration

One cubic centimeter of the ash solution is transferred to a 25-cc. conical Pyrex glass centrifuge tube. These tubes should be used for no other purposes and must be washed with water and a brush only. Two drops of 10 per cent sodium-free potassium hydroxide are added and thoroughly mixed with the contents of the tube by means of a footed glass stirring rod; 5 cc. of pyroantimonate are added and mixed with the solution in the same manner. 1.5 cc. of 95 per cent alcohol is then introduced slowly, with constant stirring. The tube is set aside for thirty minutes and then centrifuged for five minutes. The supernatant fluid is decanted and the tube is drained by inverting it on a mat of filter paper. The precipitate is washed with 5 cc. of 30 per cent alcohol, the mixture again centrifuged, the supernatant fluid decanted and the tube drained as before. The precipitate is dissolved in 2.5 cc. of 10 N hydrochloric acid and 2.5 cc. of water. Then 2 cc. of 2 per cent potassium iodide are added and the solution is titrated at once with 0.1 N sodium thiosulfate until the iodine color disappears. Starch may be used, if desired; but the end point is so sharp that it is unnecessary.

Calculation

Each cubic centimeter of 0.1 N thiosulfate is theoretically equivalent to 1.15 mg. of Na. If the theoretical factor is used, however, the method yields, as Bálint (2) showed, about 103 per cent of the sodium present. Therefore the factor $\frac{1.15}{1.03} = 1.117$ is used.

$$1.117\ (A - B) = \text{milligrams of Na in sample.}$$

$$\frac{1.117\ (A - B)}{V} \quad \text{grams of Na per liter of urine or serum.}$$

$$\frac{111.7\ (A - B)}{V} \quad \text{milligrams of Na per 100 cc. of serum.}$$

$$\frac{48.6\ (A - B)}{V} = \text{milli-equivalents of Na per liter of serum or urine.}$$

A and B indicate the cubic centimeters of 0.1 N thiosulfate used in the titration of the unknown solution and the blank respectively, and V the cubic centimeters of serum or urine represented in the sample.

Remarks

The proportions of reagents prescribed are satisfactory for the determination of from 2.00 to 3.75 mg. of Na. The Na content of 1 cc. of serum even in extreme pathological cases falls within this range. The volume of the reaction mixture can be varied within limits, as desired; but the proportions of the reagents in the reaction mixture must always be the same. A blank determination must be made on all the reagents with 0.1 N HCl substituted for the solution of serum ash. It is quite feasible, as Rourke (20) has pointed out, to use smaller amounts of serum for an analysis, titrating with 0.05 N thiosulfate.

MICRO COLORIMETRIC SODIUM METHOD

Yoshimatsu (30), as part of his system for the micro determination of the inorganic constituents of serum, has devised a colorimetric method for the determination of sodium in 0.1 cc. of serum. The method depends upon the precipitation and isolation of sodium as the pyroantimonate, the reduction of the antimonate in acid solution by sodium sulphide and the comparison of the orange-red color thus produced with that of a standard sodium solution similarly treated.

PLATINIC CHLORIDE MICRO TITRATION AND COLORIMETRIC METHODS FOR POTASSIUM. BLOOD, URINE, AND STOOLS. SHOHL AND BENNETT (22)

Both titration and colorimetric methods utilizing the chloroplatinate have been described by Shohl and Bennett (22). The potassium chloroplatinate is converted by addition of potassium iodide into the wine colored iodoplatinate. This can be estimated colorimetrically by comparison with known solutions of chloroplatinate similarly treated, or can be titrated with thiosulfate. One cubic centimeter of 0.01 N thiosulfate titrates 0.01 m.-Eq. or 0.391 mg. of K.

$$K_2PtI_6 + 2\ Na_2S_2O_3 = K_2PtI_4 + 2\ NaI + Na_2S_4O_6.$$

The colorimetric procedure was first used by Cameron and Failyer (8) for the determination of potassium in water.

Compared with micro methods dependent on precipitation of potassium as the cobaltinitrite, chloroplatinate methods have the advantage that they are based on a precipitate of definite and constant composition. In the experience of the authors the chloroplatinate micro methods are to be preferred. One can not, however, claim even for them the same reliability as for the macro gravimetric chloroplatinate or perchlorate method, and the macro procedures should be used whenever maximum accuracy is desired and the necessary amount of material is available.

The accuracy of the Shohl and Bennett methods is such that 0.1 mg. of K (the amount in 0.5 cc. of serum) can usually be determined with an error within ±4 per cent, and 0.4 mg. with an error within ±2 per cent.

Reagents

Trichloroacetic acid, 20 per cent.

Hydrogen peroxide, 30 per cent ("Superoxol," Merck, Blue Label).

Sulfuric acid, approximately 4 N.

Hydrochloric acid, approximately 1 N.

Chloroplatinic acid, containing 10 per cent of platinum; 26.5 grams of chloroplatinic acid, $H_2P_tCl_6 \cdot 6H_2O$ dissolved in water and diluted to 100 cc.

Absolute alcohol, redistilled over lime.

Absolute alcohol, redistilled over lime and saturated with potassium chloroplatinate by shaking the alcohol with a small quantity of the salt, at intervals, for several days.

Potassium chloride, 10 per cent, saturated with potassium chloroplatinate in the same manner.

Potassium iodide, 2 N.

Standard potassium solution for colorimetric determination. Dissolve 8.914 grams of dry potassium sulfate in water and dilute it to a liter; 1 cc. of this solution contains 4 mg. of K. From this solution, standards for use can be prepared when required, by dilution. The most useful standard for blood and serum is one that contains 0.2 mg. in 5 cc. (1 volume of stock standard solution diluted to 100 volumes with water); 5 or 10 cc. of this standard are used for a determination.

Special solutions for volumetric determination. Sodium thiosulfate 0.01 N standardized daily against potassium iodate or biiodate (see p. 33).

Hydrogen peroxide, 0.2 per cent, made up just before it is to be used.

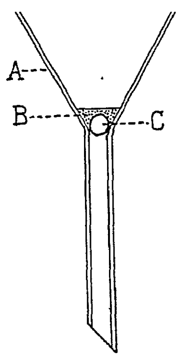


FIG. 85. Micro filter for potassium platinic chloride

Preparation of material

The serum or blood may be prepared either by ashing or by removing the proteins with trichloroacetic acid.

Precipitation of blood proteins. (a) ***For blood serum.*** To 1 volume of serum, mixed with 3 volumes of water, is added 1 volume of 20 per cent trichloroacetic acid. The mixture is allowed to stand a few minutes and filtered through an ash-free filter. Filtration is facilitated and the yield of filtrate is increased by preliminary centrifugation. Five cubic centimeters of filtrate, equivalent to 1 cc. of serum or plasma, are taken for the colorimetric technique, twice as much for volumetric determination.

(b) ***For whole blood.*** To 1 volume of whole blood, citrated or defibrinated, laked by admixture with 2 volumes of water, are added 2

volumes of 20 per cent trichloroacetic acid. The mixture is allowed to stand for a few minutes and then centrifuged and filtered. One cubic centimeter of the filtrate, equivalent to 0.2 cc. of blood, is taken for the colorimetric technique, twice as much for the volumetric determination.

Ashing serum or plasma. An amount of the sample containing from 0.15 to 0.80 mg. of potassium is transferred to a large Pyrex glass test tube, 25 by 200 mm. 0.6 cc. of 4 N sulfuric acid is added and the mixture is evaporated to a small volume. To the residue, at intervals, a drop or more of 30 per cent hydrogen peroxide is added and boiling is continued until a clear, colorless solution is obtained while sulfuric acid fumes are escaping. Care should be taken that all the hydrogen peroxide has been expelled before the procedure is continued. The sample is then transferred with the aid of a small amount of water to a platinum crucible or dish, in which it is evaporated to a small volume, and ignited as described for "Ignition of alkali sulfates" on page 720 of the total base chapter.

Ashing of whole blood, urine, or feces. Urine and feces are first ashed by the Stolte process (see p. 70). The ash is taken up in a small amount of 0.5 N hydrochloric acid. Whole blood is freed of protein by means of trichloroacetic acid as described above.

An amount of ash solution or blood filtrate (1 to 2 cc. of the latter) containing from 0.15 to 0.80 mg. of K is transferred to a platinum dish or crucible. Four drops of 4 N sulfuric acid are added and the mixture is concentrated and ignited as described for "Ignition of alkali sulfates" on page 720 of the total base chapter. The sulfates are redissolved and transferred to a small glass or porcelain evaporating dish with a minimal amount of water.

Precipitation of potassium chloroplatinate. The solution of alkali sulfates is evaporated to dryness on a steam bath. To the residue are added 1 drop of 1 N hydrochloric acid and 0.30 cc. of chloroplatinic acid. When this has been thoroughly mixed, 5 cc. of alcohol are introduced. After the mixture has stood for twenty minutes, the precipitate is transferred to a Shohl (21) micro filter. This (see fig. 85) is made by mounting a 1-inch funnel in a Witt filtering apparatus, which is a suction flask with a ground glass, removable top, devised so that the filtrate may be recovered in a small inner tube. A glass pearl is dropped into the funnel and a mat of fine grained asbestos about $\frac{1}{32}$-inch thick is packed over the bead. This makes essentially a micro Caldwell

crucible. The excess platinum is filtered off by suction and saved for recovery. Precipitate and filter are then washed 4 or 5 times with alcohol saturated with potassium chloroplatinate. Some contaminating salts, which are precipitated with the chloroplatinate in alcohol, are washed out with 3 or 4 portions of 10 per cent potassium chloride saturated with potassium chloroplatinate.

Determination of potassium chloroplatinate

(a) ***Colorimetric method.*** A test tube is placed in the suction flask under the micro filter, and the precipitate is dissolved *in situ* by the repeated addition of small amounts of hot water, which are first used to rinse the platinum crucible. The washings caught in the test tube are transferred to a 25-cc. volumetric flask. Five cubic centimeters of 2 N potassium iodide and 1 cc. of 1 N hydrochloric acid are then added, the mixture is cooled and diluted to volume. The deep wine-red color can be compared at once against known potassium sulfate standards, containing 0.2 to 0.4 mg. of K per 5 cc., which have been subjected to the same procedure as the unknown, throughout. This obviates the necessity of introducing a blank correction for impurities in reagents.

(b) ***Titrimetric method.*** The funnel containing the precipitate is removed from the filtering apparatus and inverted. The precipitate, together with the asbestos, is returned to the original dish by inserting a small glass rod in the stem of the funnel. The sides of the funnel are washed with 1 to 2 cc. of hot water and 1 cc. of 2 N potassium iodide. The mixture is heated in a water bath at 65° for fifteen minutes. The solution is then titrated, while still hot and without removal of the asbestos, with 0.01 N sodium thiosulfate delivered from a microburette with 0.01 or 0.02 cc. divisions (see p. 13). The end-point is a lemon-yellow color free from red. A blank determination must, of course, be made by carrying out the whole procedure with water as the unknown solution.

(c) ***Combination titrimetric and colorimetric method.*** The volumetric result can be checked colorimetrically by reoxidation of the solution. One cubic centimeter of 1 N hydrochloric acid and 0.10 cc. of a fresh 0.2 per cent hydrogen peroxide solution are added to the mixture after the titration has been completed. After this has stood exposed to the air from thirty to sixty minutes, it is diluted to 50 cc. and compared with a suitable colorimetric standard.

Calculations

Colorimetric method.

$$\frac{n\ S}{U} = \text{milligrams of K in sample analyzed} = a.$$

$$\frac{a}{V} = \text{grams of K per liter of material.}$$

$$\frac{100\ a}{V} = \text{milligrams of K per 100 cc. of material.}$$

$$\frac{25.6\ a}{V} = \text{milli-equivalents of K per liter of material.}$$

S and U represent colorimeter readings of standard and unknown respectively; n = milligrams of K in standard; V = cubic centimeters of blood, serum, or urine represented by the sample

Volumetric method.

$$0.391\ (A - B) = \text{milligrams of K in sample.}$$

$$\frac{0.391\ (A - B)}{V} = \text{grams of K per liter of material.}$$

$$\frac{39.1\ (A - B)}{V} = \text{milligrams of K per 100 cc. of material.}$$

$$\frac{10\ (A - B)}{V} = \text{milli-equivalents of K per liter of material.}$$

A and B represent cubic centimeters of 0.01 N thiosulfate used for the titration of the unknown solution and the blank respectively; V = volume of material represented by the sample analyzed.

Precautions

Access of ammonia fumes during precipitation and washing of the potassium chloroplatinate must be avoided, as ammonium forms an insoluble chloroplatinate. For the same reason care must be taken that all ammonia is expelled from the ash. Sulfuric acid ashing should be carried out on all samples *just before* the precipitation with chloroplatinic acid to insure the expulsion of any ammonia which may have beem absorbed.

Ashing is an essential part of the procedure for all materials which contain

ammonia and other nitrogenous compounds, including even the most minute quantities of protein.

COBALTINITRITE METHODS

The potassium is precipitated and isolated as the potassium sodium cobaltinitrite of the approximate composition $K_2NaCo(NO_2)_6$, and the nitrite in the precipitate is titrated by permanganate.

$$5\ HNO_2 + 2\ KMnO_4 + 3\ H_2SO_4 = 5\ HNO_3 + K_2SO_4 + 2\ MnSO_4 + 3\ H_2O$$

The first micro potassium method used for blood analyses, and the one by which most of the blood potassium analyses in the literature at present have been obtained, is the application of Adie and Wood's cobaltinitrite titration procedure to blood devised by Clausen (9) and further developed by Kramer and Tisdall (15, 17, 26).

Sodium cobaltinitrite, $Na_3Co(NO_2)_6$, reacts with potassium salts to form an insoluble compound in which the sodium in the molecule is partly replaced by potassium. Under the conditions usually employed the precipitate approximates the composition $K_2NaCo(NO_2)_6 \cdot nH_2O$, the value of n being 1 or greater according to the temperature and duration of precipitation. The precipitate can be measured by weight or by any procedure for determining either the cobalt or the nitrite in it. Such methods, titrimetric, colorimetric, and gasometric, accurate and convenient for microanalyses, are available, and would afford ideal micro procedures if the cobaltinitrite could be obtained with constant composition.

Ever since Adie and Wood introduced the method in 1900 it has been the object of work and controversy. Its attractive possibilities have lured one investigator after another to seek conditions under which a precipitate of constant composition could be obtained. Some have reported success while others gave up in disgust. Thus Strecker and Jungck (24) in 1923 after a careful review of the literature and experimental studies of their own were unable to find conditions under which a precipitate was formed of constant composition. From 0.500 gram of KCl precipitated with a constant amount of cobaltinitrite in the same volume of solution they obtained precipitates varying from 1.651 to 1.501 grams, the theoretical being 1.523 grams for $K_2NaCo(NO_2)_6 \cdot H_2O$. Recent papers by Bonneau (4) and Van Rysselberge (27) give the literature on the controversy and report experimental work which appears to place the cobaltinitrite methods on a somewhat more secure basis than formerly. The variation in the weight of the precipitate appears to be in part due to variation in the water of hydration. According to Bonneau, the rest of the molecule has the constant composition

$K_2NaCo(NO_2)_6$ if when the precipitate is formed the ratio Na:K in the solution exceeds 22 (the Na in the sodium cobaltinitrite precipitant was included in calculating the Na:K ratios). If the solution in which the precipitate formed contained more than 1/22 as much K as Na the precipitate tended more towards the composition $K_3Co(NO_2)_6$, and when the ratio Na:K was lowered to 1 the precipitate was almost entirely $K_3Co(NO_2)_6$. Bonneau recommends precipitating from solutions where the ratio Na:K is from 25 to 100. In his experiments all precipitations were done at nearly 100°, sodium nitrite and afterwards cobalt nitrite being added to the potassium solution instead of adding the cobalt and sodium nitrites together in a previously mixed solution, as is usually done.

Both Bonneau and Van Rysselberge agree that the factor for calculating potassium from the amount of cobaltinitrite precipitate should be determined by each analyst by control analyses done with the same conditions for precipitation and approximately the same amounts of potassium and the same Na:K ratios which occur in the unknown solutions analyzed. Van Rysselberge states that if calculation factors are thus empirically determined by analyses of a series of known solutions covering the variations of K content and Na:K ratios encountered in the unknowns, potassium determinations can be made with an error not exceeding 1.3 per cent.

In normal blood serum the K content and the Na:K ratio vary but little, and it appears that accurate potassium determinations should be possible if the calculation factors are determined by analysis of similar known solutions. This in fact was done by Clausen (9) and Kramer and Tisdall (15), and their figures for the potassium content of normal human serum are practically the same as those obtained with the classical macro gravimetric methods. To judge from their controls the same calculation factor sufficed also to cover the maximum pathological variations of serum potassium content. How accurately the same factor holds for the more variable range of K content and Na:K ratios found in stools and urine is uncertain.

In place of the potassium sodium cobaltinitrite, Breh and Gaebler (5) have recently introduced precipitation of the potassium *silver* cobaltinitrite, previously described by Burgess and Kamm (7). This salt has an advantage in that it is more insoluble in water than the sodium salt, and consequently the precipitation can be made directly in the dilute Folin-Wu filtrate. This procedure, published in 1930, has unfortunately not had time to be put to the test of routine analysis. If it yields a precipitate of more constant composition as well as greater insolubility than the sodium cobaltinitrite method, it may be the improvement that is needed in order to lift the cobaltinitrite methods to a place among precise potassium determinations.

COBALTINITRITE MICRO TITRATION OF POTASSIUM. CLAUSEN (9), KRAMER AND TISDALL (15)

Reagents

Sodium cobaltinitrite reagent. *A*. Dissolve 25 grams of cobalt nitrite crystals in 50 cc. of water and add 12.5 cc. of glacial acetic acid. *B*. Dissolve 120 grams of sodium nitrite, (potassium-free, Merck's reagent) in 180 cc. of water.

Add 210 cc. of *B* to the whole of *A* and draw air through the solution until all nitric oxide gas has been driven off. The reagent is placed in the refrigerator, where it will keep for a month. It is filtered each time before it is used.

1:2 Sodium nitrite solution. Dissolve 50 grams of potassium-free Merck's "Reagent" sodium nitrite in 100 cc. of water.

Potassium permanganate. 0.02 N freshly made by dilution from 0.1 N and titrated daily against 0.01 N sodium oxalate (for preparation and standardization of permanganate, see page 31).

Sodium oxalate (Sörensen salt), 0.01 N.

Preparation of material

Urine and feces must be ashed before the cobaltinitrite precipitation is applied, because ammonia and other organic constituents would interfere. For serum Kramer and Tisdall either used the ash (15) or added the cobaltinitrite directly to the serum. They found no difference in the results. Taylor (25), in a recent paper uses only the protein-free tungstic acid filtrate, and Breh and Gaebler (5) found that when the direct precipitation was applied to dog sera protein was absorbed by the precipitate. No data are given, however, to indicate how much error in the permanganate titration was caused by the absorbed protein. At present the analyses of Kramer and Tisdall (16), showing results by direct precipitation agreeing within the limit of error of the method with the results obtained by analyses of ashed serum, stand without contradiction. The analyst may therefore, apply the following method either to the whole serum or plasma or to its ash.

Precipitation with cobaltinitrite (17)

In a 15-cc. graduated centrifuge tube is placed an amount of solution containing 0.2 to 0.5 mg. of potassium. This amount is represented in 1 cc. of serum or its ash, in the ash of 0.1 to 0.2 cc. of whole blood, in about 1/1000th of the twenty-four-hour feces of an adult, or

1/10,000th of the twenty-four-hour urine. The material should be dissolved in not more than 1 cc. of water and may contain free mineral acid equivalent to not more than 1 cc. of 0.1 N solution. Then 0.5 cc. of the 1:2 sodium nitrite solution is added, and the contents of the tube are thoroughly mixed and permitted to stand five minutes.[5] Water is added to make the volume up to 4 cc. and the contents are again mixed. Two cubic centimeters of the sodium cobaltinitrite reagent are then added ***slowly drop by drop,*** each drop being mixed as added with the solution in the tube. If the reagent is added rapidly a plus error exceeding 10 per cent may be caused by occlusion of nitrite in the precipitate. The contents of the tubes are allowed to stand for half an hour, and are then centrifuged for seven minutes at 1300 revolutions per minute. All but 0.2 or 0.3 cc. of the supernatant fluid is removed. The removal is accomplished by means of a tube drawn out to a capillary of about 1-mm. diameter and curved upward in U form at the end. It may be fixed in a 2-hole stopper, and air pressure exerted through a tube entering the other hole, so that the fluid is blown out. Or the fluid may be drawn off by controlled suction. Five cubic centimeters of water are allowed to run down the sides of the tube, which is then gently agitated so that the water is mixed with the residual fluid above the precipitate, but without disturbing the precipitate. The agitation may be accomplished by holding the tube vertically and gently hitting the lower end with a circular motion. The brown fluid may be seen to rise and mix with the water. The tube is then centrifugated for five minutes. The procedure is repeated 3 times, so that the precipitate is washed with 4 portions of water. The supernatant fluid from the last washing should be perfectly clear. After the removal of the final washing fluid the precipitate is ready to be titrated.

Titration

An excess of 0.02 N potassium permanganate is added (1.6 to 2 cc. are sufficient for normal blood), followed by 1 cc. of approximately 4 N sulfuric acid. The precipitate is then thoroughly mixed with the fluid by means of a glass rod. The sample is heated in the boiling water

[5] If the sodium nitrite is not added, it will be found that the precipitate obtained on the addition of the cobaltinitrite reagent will float on the surface of the fluid and adhere to the sides of the tubes. The precipitate will also adhere to the sides unless the tubes have been previously cleaned with the use of a brush, washed out with a strong cleaning fluid (commercial H_2SO_4 and dichromate) and then thoroughly rinsed with distilled water. Low results will be obtained unless these precautions are observed.

bath for forty-five to sixty seconds at the end of which time the solution should be clear and still pink. If all the precipitate is not oxidized, the contents will be cloudy and the intensity of the color will be seen to diminish. Heating should then be continued until the solution is clear but still pink. When the heating is continued too long, the contents again become cloudy and have a brownish color. If this is allowed to happen, the sample must be discarded, as high results will be obtained. An amount of 0.01 N sodium oxalate sufficient to decolorize the solution completely (generally 2 cc.) is promptly added. The excess of oxalate is then determined by titrating to a definite pink color with 0.02 N potassium permanganate delivered from a micro-burette graduated in 0.02 cc.

Calculation

$$f\,(2\,[A - C] - B) = \text{milligrams of K in sample} = a.$$

f is a *factor* representing the milligrams of K indicated by 1 cc. of 0.01 N permanganate in the titration. The value of f found by Kramer and Tisdall was 0.071, but it is desirable that the analyst should determine it himself with his own reagents as described below.

A is the total cubic centimeters of 0.02 N permanganate used in the titration.

C is the cubic centimeters of 0.02 N permanganate required to give a visible color with a volume of water equal to the volume of the titrated solution.

B is the cubic centimeters of 0.01 N oxalate used in the titration.

$$\frac{a}{V} = \text{grams of K per liter of urine.}$$

$$\frac{100\,a}{V} = \text{milligrams of K per 100 cubic centimeters of serum.}$$

$$\frac{1000\,a}{39.1\,V} = \frac{25.6\,a}{V} = \text{milli-equivalents of K per liter of serum or urine.}$$

V is the number of cubic centimeters of urine or serum represented in the sample.

Example. Two cubic centimeters of 0.02 N potassium permanganate are originally added and 0.43 cc. of the same solution is used in the final titration. Two cubic centimeters of 0.01 N oxalate are used to decolorize the solution after the first addition of permanganate. The volume of permanganate required to give a visible color to 4.4 cc. of water is 0.03 cc. Hence, if we use 0.071 as the value of f, the calculation is:

$$\text{Milligrams of K in sample} = 0.071\,(2\,[2.43 - 0.03] - 2.00) = 0.199 \text{ mg.}$$

Determination of factor, f. Theoretically, if the precipitate were $K_2NaCo(NO_2)_6$, 1 cc. of permanganate would be equivalent to 0.065 instead of 0.071 mg. of potassium. The higher factor indicates that in the precipitate as formed the ratio of $K:NO_2$ is greater than 2:6, and that a small fraction of the precipitate is $K_3Co(NO_2)_6$ instead of $K_2NaCo(NO_2)_6$. Those who have most recently studied the cobaltinitrite method (4, 27) are in agreement that each analyst must determine for himself under the conditions of his analyses the potassium equivalent of the permanganate with which he titrates. In the present case this equivalent can be determined by analyses of solutions containing 0.2 and 0.4 mg. of potassium per cc., or 0.3815 and 0.783 mg. of KCl. The factor is calculated as

$$f = \frac{\text{milligrams of K}}{2\ (A - C) - B}$$

where f, A, B, and C have the same significance as in the preceding calculation.

COBALTINITRITE MICRO COLORIMETRIC POTASSIUM DETERMINATION. SILVER COBALTINITRITE. METHOD OF BREH AND GAEBLER (5)

This method was developed primarily for analysis of Folin-Wu serum filtrate. The potassium is precipitated in the presence of a silver salt and sodium cobaltinitrite. The precipitate formed is potassium silver cobaltinitrite, which is even less soluble than potassium sodium cobaltinitrite. Consequently, whereas the latter salt can not be precipitated quantitatively in the Folin-Wu blood filtrate until the latter has been concentrated to a small volume, the silver salt can be precipitated directly in the filtrate as obtained. The insolubility of the silver salt also renders it comparatively immune to loss when washed with water. The presence of the silver makes the precipitate unsuitable for titration with permanganate, but it can be measured colorimetrically, or according to the authors, by the gasometric method of Kramer and Gittleman described on page 430 of the gasometric chapter.

Reagents

1. *Potassium reagent.* To 20 cc. of sodium cobaltinitrite reagent described above on page 748 add 2 cc. of 40 per cent silver nitrate solution. Shake vigorously. Filter to remove the trace of precipitate which fails to redissolve.

2. *Dilute nitric acid.* A solution prepared by diluting 20 cc. of concentrated nitric acid (specific gravity 1.42) to 100 cc. with water.

3. *Ammonium thiocyanate solution.* A freshly prepared 2 per cent solution of the chemically pure salt in 95 per cent alcohol.

4. *Potassium standard.* An aqueous solution of potassium sulfate containing 1 mg. of potassium per cubic centimeter, prepared by dissolving 2.229 grams of the salt and diluting to 1 liter. This is a stock solution. For the standard dilute 5 cc. of this to 100 cc., making a solution containing 0.25 mg. of potassium in 5 cc.

5. *Silver nitrate solution.* A 5 per cent aqueous solution.

6. *Protein precipitants.* A 10 per cent solution of sodium tungstate, and a $\frac{2}{3}$ N solution of sulfuric acid.

Procedure

Place 2 cc. of serum in a 15-cc. centrifuge tube. Add 5 cc. of water, and 1 cc. of sodium tungstate solution. Mix. Add 1 cc. of $\frac{2}{3}$ N sulfuric acid. Stopper and shake well. Add 1 cc. of the 5 per cent silver nitrate solution, bringing the total volume up to 10 cc., and shake again. After fifteen minutes standing centrifuge. The precipitate should occupy considerably less volume than the supernatant liquid. The latter may be filtered through a very small ash-free paper or may be removed with a pipette. When the pipette is inserted into the liquid the upper opening of the pipette is closed with the finger so that any film on the surface of the liquid will adhere to the outside of the pipette.

In two 15-cc. graduated conical Pyrex centrifuge tubes, which have been cleaned previously with sulfuric acid-bichromate mixture,[6] place 5 cc. of the potassium standard (0.25 mg. of K) and 5 cc. of serum filtrate respectively. Pass the tube with the standard solution through the flame a few times so that it becomes barely warm to the hand.[7] Then add 2 cc. of the potassium reagent to each tube. After two hours standing centrifuge for fifteen minutes at a speed of about 1200 revolutions per minute. With a capillary siphon, the tip of which bends upward, siphon off the supernatant liquid to the 0.3 cc. mark. Wash three or four times with 5-cc. portions of distilled water, as in the Kramer-Tisdall method. Centrifuge for five minutes each time, and

[6] Tubes which have been cleaned and dried should, before being used, be rinsed to remove any trace of ammonium salt, which forms a highly insoluble cobaltinitrite.

[7] The silver compound will otherwise precipitate very rapidly from standards containing over 0.15 mg. of potassium in 5 cc. and will form a fine yellow precipitate which adheres to the tube instead of a coarser one which is reddish after centrifuging.

siphon off the washing to the 0.3-cc. mark. The wash water should be mixed well with the residual solution above the precipitate, but the latter is stirred up as little as possible. The last washing should be free of yellow color. In the case of the last siphoning especial care should be taken to remove the supernatant liquid exactly to the 0.3-cc. mark. After removal of the wash water add 1 cc. of the dilute nitric acid to each tube, and heat carefully to boiling over a micro burner. Just as boiling begins remove the tube from the flame and shake gently. In ten to 20 seconds the yellow precipitate dissolves completely. Should any trace remain it can be dissolved by continuing the heat below the boiling point, and shaking several times. Cool the tubes under the tap, add alcoholic thiocyanate solution to the 8-cc. mark, mix, and compare in the colorimeter. It is well to rinse cups and plungers with alcohol acidified with nitric acid before using them. As is the rule with alcoholic solutions, minute bubbles under the plunger must be watched for and removed.

Calculation

The theoretical formula is:

$$\text{Milligrams of K in sample} = 0.25 \times \frac{S}{U}$$

S and U represent the readings of standard and unknown respectively. 0.25 mg. of K is present in the standard. Breh and Gaebler found that this formula held exactly only when the K content of the unknown was between 0.20 and 0.30 mg. Therefore they constructed the empirical curve shown in figure 86, by means of which results can be calculated for analyses of samples containing from 0.15 to 0.50 mg. of K, about the extreme range encountered in blood serum.

From milligrams of K in sample one may calculate grams of K per liter, milligrams per 100 cc., or milli-equivalents per liter as described for calculation of results by the Kramer-Tisdall method above.

Construction of empirical curve. From the stock standard potassium solution with 1 mg. per cubic centimeter, a series of 8 solutions containing from 0.15 to 0.80 mg. of K per 5 cc. portion is prepared. Several series of determinations are carried out on these solutions, using 5 cc. of each, and taking the one containing 0.25 mg. of potassium as standard. The average readings obtained on the other solutions are plotted in a curve in which milligrams of K serve as ordinates and readings as abscissae.

Alternative colorimetric measurement of the precipitate by diazo reaction

The filtrate is prepared from 1 cc. of serum, 7.5 cc. of water, 0.5 cc. of 10 per cent sodium tungstate, 0.5 cc. of $\frac{2}{3}$ N sulfuric acid, and 0.5 cc.

of 5 per cent silver nitrate solution. Precipitation and washing are carried out in the manner described above, with 5 cc. of filtrate. The standard is 5 cc. of potassium sulfate solution containing 0.08 mg. of potassium in this volume. After the washing has been completed 5 cc. of 0.2 N sodium hydroxide solution are added to each tube. The contents are heated to boiling, filtered from precipitated cobalt and silver hydroxides into 50 cc. volumetric flasks, and made up to volume by washing. Eight-cubic centimeter portion of standard and unknown are transferred to 100-cc. volumetric flasks, and diluted to about 70 cc. Two cubic centimeters of sulfanilic acid solution (0.5 per cent in 30

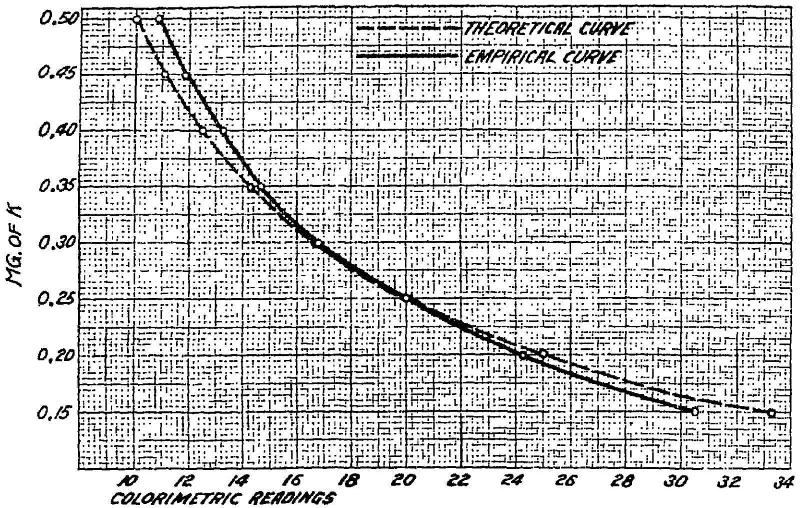


FIG. 86. Curve for calculation of potassium by Breh and Gaebler's method. Abscissae represent colorimetric readings of the unknown solution when standard, prepared with 0.25 mg. of K, is set at 20 mm.

per cent acetic acid) and 1 cc. of naphthylamine solution (0.5 per cent in 30 per cent acetic acid) are added. After dilution to volume and ten minutes standing comparison is made in the colorimeter. The formula

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 16$$

gives the value in milligrams of potassium per 100 cc. If the reading is too far from the standard there is ample solution left to repeat the final

determination with a different aliquot of the contents of the 50-cc. volumetric flask.

The colorimetric diazo procedure, first published for potassium determinations by Briggs (6), is accredited by him to unpublished work of Bell and Doisy. It has for micro potassium analyses an advantage over the preceding colorimetric cobalt determination in that the diazo method requires less serum and yields a larger volume of colored solution for use in the colorimeter.

MICRO ELECTROLYTIC SODIUM PLUS POTASSIUM DETERMINATION

Stoddard (23) removes the alkaline earths, as in the classical methods, and then submits the filtrate containing Na and K salts to electrolysis with a mercury cathode. The Na and K amalgamate with the mercury, from which they are extracted by boiling with excess of standard hydrochloric acid, and determined by alkalimetric titration.

BIBLIOGRAPHY

1. Adie, R. H., and Wood, T. B.: A new method of estimating potassium. *J. Chem. Soc.*, 1900, **77**, 1076.
2. Bálint, M.: Eine jodometrische Mikrobestimmung des Natriums. *Biochem. Z.*, 1924, **150**, 425.
3. Barber, H. H., and Kolthoff, I. M.: A specific reagent for the rapid gravimetric determination of sodium. *J. Am. Chem. Soc.*, 1928, **50**, 1625.
4. Bonneau, L.: Recherches sur la composition du cobaltinitrit de potassium-sodium. *Bull. soc. chim. France*, 1929, (Series IV) **45**, 798.
5. Breh, F., and Gaebler, Oliver, H.: The determination of potassium in blood serum. *J. Biol.Chem.*, 1930, **87**, 81.

5a. Butler, A. M., and Tuthill, Elizabeth: An application of the uranyl zinc acetate method for determination of sodium in biological material. *J. Biol. Chem.*, 1931, **93**, 171.

6. Briggs, A. P.: A study of the inorganic elements of blood plasma. *J. Biol. Chem.*, 1923, **57**, 351.
7. Burgess, L. L., and Kamm, O.: A study of cobaltinitrites and their application to analytical chemistry. A. Silver-potassium cobaltinitrites: A new qualitative test for potassium. *J. Am. Chem. Soc.*, 1912, **34**, 652.
8. Cameron, F. K., and Failyer, G. H.: The determination of small amounts of potassium in aqueous solutions. *J. Am. Chem. Soc.*, 1903, **25**, 1063.
9. Clausen, S. W.: A method for estimation of potassium in the blood. *J. Biol. Chem.*, 1918, **36**, 479.
10. Kolb, A., and Formhals, R.: Über die Einwirkung von Kaliumjodid und Salzsäure auf Antimonsäure. *Z. anorg. Chem.*, 1908, **58**, 189.
11. Kolb, A., and Formhals, R.: Die titrimetrische Bestimmung des Antimons. *Z. anorg. Chem.*, 1908, **58**, 202.
12. Kolthoff, I. M.: Ein spezifisches Reagens auf Natrium. *Z. anal. Chem.*, 1927, **70**, 397.

13. KRAMER, B.: Direct quantitative determination of potassium and sodium in small quantities of blood. *J. Biol. Chem.*, 1920, **41**, 263.
14. KRAMER, B., AND GITTLEMAN, I.: An iodometric method for the determination of sodium in small amounts of serum. *J. Biol. Chem.*, 1924, **62**, 353.
15. KRAMER, B., AND TISDALL, F. F.: A clinical method for the quantitative determination of potassium in small amounts of serum. *J. Biol. Chem.*, 1921, **46**, 339.
16. KRAMER, B., AND TISDALL, F. F.: A simple method for the direct quantitative determination of sodium in small amounts of serum. *J. Biol. Chem.*, 1921, **46**, 467.
17. KRAMER, B. AND TISDALL, F. F.: The direct quantitative determination of sodium, potassium, calcium and magensium in small amounts of blood. *J. Biol. Chem.*, 1921, **48**, 223.
18. LEULIER, A., VELLUZ, L., AND GRIFFON, H.: Sur le microdosage du potassium, sous forme de cobaltinitrite. *Bull. soc. chim. biol.*, 1928, **10**, 891.
19. Official and tentative Methods of Analysis of the Association of Official Agricultural Chemists, Washington, 1st Edition, 1920, page 12.
20. ROURKE, M. D.: On the determination of the sodium content of small amounts of serum or heparinized plasma by the iodometric method. *J. Biol. Chem.*, 1928, **78**, 337.
21. SHOHL, A. T.: Apparatus for micro-filtration. *J. Am. Chem. Soc.*, 1928, **50**, 417.
22. SHOHL, A. T., AND BENNETT, H. B.: A micro method for the determination of potassium as iodoplatinate. *J. Biol. Chem.*, 1928, **78**, 643.
23. STODDARD, J. L.: An electrolytic method for the determination of sodium plus potassium. *J. Biol. Chem.*, 1927, **74**, 677.
24. STRECKER, W., AND JUNGCK, A.: Über die gewichtsanalytische und titrimetrische Bestimmung des Kaliums. *Z. anal. Chem.*, 1923, **63**, 161.
25. TAYLOR, F. H. L.: The determination of potassium in blood serum. *J. Biol. Chem.*, 1930, **87**, 27.
26. TISDALL, F. F., AND KRAMER, B.: Methods for the direct quantitative determination of sodium, potassium, calcium and magnesium in urine and stools. *J. Biol. Chem.*, 1921, **48**, 1.
27. VAN RYSSELBERGE, P. J.: Quantitative determination of potassium by sodium cobaltinitrite method. *Ind. and Eng. Chem.* (Anal. Ed.) 1931, **3**, 3.
28. VAN SLYKE, D. D.; WU. H., AND MCLEAN, F. C.: Studies of gas and electrolyte equilibria in blood. V. Factors controlling the electrolyte and water distribution in the blood. *J. Biol. Chem.*, 1923, **56**, 765.
29. WAKEMAN, A. M.; EISENMAN, A. J., AND PETERS, J. P.: A study of human red blood cell permeability. *J. Biol. Chem.*, 1927, **73**, 567.
30. YOSHIMATSU, S.: A colorimetric method for determination of potassium with 0.2 cc. of blood. *Tohoku, J. Exp. Med.*, 1926, **8**, 174.
31. YOSHIMATSU, S.: Colorimetric method for the determination of sodium with 0.1 cc. of serum or blood. *Tohoku, J. Exp. Med.*, 1927, **8**, 496.

CHAPTER XXV

CALCIUM

DISCUSSION

General calcium methods

Nearly all calcium determinations, both in clinical and general mineral analyses, involve precipitation of Ca as the very insoluble oxalate. The latter can be determined by several procedures.

1. When the oxalate is heated to 300° it loses one molecule of CO and changes to the carbonate.

$$CaC_2O_4 = CaCO_3 + CO$$

The $CaCO_3$ can be weighed.

2. However, if overheated some decomposition of the carbonate to the oxide occurs. Therefore the classical gravimetric method has been to ignite at white heat in platinum until all the CO_2 has been driven off, and weigh the oxide.

$$CaC_2O_4 = CaO + CO + CO_2$$

3. Instead of weighing, either the carbonate or oxide or a mixture of them obtained by ignition in platinum can be dissolved in excess of standard hydrochloric acid and titrated as an alkali.

4. Another volumetric method is offered by redissolving the oxalate precipitate in hot dilute sulfuric acid and titrating the oxalic acid with permanganate.

$$5\ H_2C_2O_4 + 2\ KMnO_4 + 3\ H_2SO_4 = 2\ MnSO_4 + K_2SO_4 + 10\ CO_2 + 8\ H_2O$$

5. A gasometric determination can be made by measuring the CO_2 evolved in the above reaction with permanganate.

Procedures 3, 4, and 5 are all adapted to rapid determinations of small amounts of calcium, and all have found use in clinical analyses. The one most used has been the permanganate titration. It is carried out without other treatment of the precipitate than washing, while the titration of the carbonate or oxide requires a preliminary ignition. The gasometric method (see p. 421) has been too recently introduced to have been greatly used, but has definite advantages, particularly for micro determinations.

Urine. The conditions for the precipitation of calcium oxalate in urine have been accurately defined by work of McCrudden (11, 12) and Shohl (16). The chief essential is that the precipitation shall occur when the pH is between 4 and 5.6. If urine is more acid than pH 4 precipitation of the oxalate will not be complete, as it begins to become appreciably soluble at more acid reactions. If the pH exceeds 5.6 some magnesium ammonium phosphate is likely to be precipitated with the calcium oxalate. McCrudden used an acetate buffer mixture to obtain the proper acidity. It can also be obtained by other buffers.

McCrudden (12) gave directions for determining the calcium in the oxalate precipitate either gravimetrically, by igniting and weighing as CaO, or volumetrically by titration with permanganate. He found that uric acid frequently contaminated the calcium oxalate precipitate, where its presence was made evident by the reddish color of the precipitate. When such contamination occurred only the gravimetric method could be used. The absorbed uric acid reacted with permanganate in the titration, so that results were too high, sometimes by as much as 50 per cent (17).

Shohl and Pedley (17) made the titration method uniformly applicable by oxidizing the organic matter of the urine with ammonium persulfate before the calcium oxalate was precipitated. The uric acid was thereby destroyed, and calcium oxalate precipitates were obtained which could always be titrated.

MacKay and Butler (unpublished work) have found that the gasometric procedure (p. 425) has for urine an advantage over titration in that the yield of CO_2 is not significantly affected by adsorbed uric acid on the calcium oxalate. The gasometric determination therefore can be done on calcium oxalate precipitated without preliminary boiling with persulfate. The final gasometric analysis is nearly as rapid as titration.

Feces. Before calcium in feces can be determined the organic matter must be destroyed. Ordinarily this is done by either wet or dry ashing. To the solution of the ash the same methods used for urine are applied. Corley and Denis (4) destroy interfering organic matter by autoclaving with dilute alkali, and then apply the oxalate precipitation and permanganate titration. Their method avoids ashing, but requires an autoclave that can be heated to 160° internal temperature.

Blood. For blood, plasma, and serum analyses most of the older analysts destroyed the proteins by ashing, and used large amounts of blood, conditions which were impediments to clinical application. In 1917 Halverson and Bergeim (5) published the first simple procedure for small amounts of blood. Their method is based on three principles:

1. The proteins are removed by precipitation in such a highly acid solution that all the calcium is kept in solution. The Folin-Wu tungstic acid precipitation of proteins can not be used. It is not acid enough to prevent loss of much calcium with the protein precipitate (2).

2. The calcium in the filtrate is precipitated according to the principles worked out by McCrudden, at a pH between 4 and 5.6.

3. The calcium oxalate precipitate is redissolved in a hot, approximately 1 N solution of sulfuric acid and titrated with permanganate of 0.01 N concentration.

In applying the micro permanganate titration to blood the principles of Halverson and Bergeim have been followed by most subsequent authors, with the exception of a number who have omitted removal of the proteins, and have precipitated the calcium by addition of oxalate directly to the more or less diluted serum. Van Slyke and Sendroy (23) showed, however, that removal of the proteins is necessary. They obtained from 5 to 15 per cent lower results when the calcium oxalate was precipitated in the presence of the proteins than when the latter were first removed with trichloroacetic acid. That the deficit in the presence of the proteins was due to unprecipitated calcium was shown by the fact that when the filtrate from the calcium oxalate was subsequently freed from proteins with trichloroacetic acid a second crop of calcium oxalate could be obtained, which brought the total up to that obtained when the entire analysis was performed in the protein-free blood filtrate.

Van Slyke and Sendroy determined the precipitated calcium oxalate both by the permanganate titration and by the micro gasometric method described on page 421, and obtained identical results, but with less error in analyses of small amounts (1 cc. of serum) when the gasometric procedure was used.

Hamilton (6), Trevan and Bainbridge (22), and Fiske and Adams (4-*a*) have heated the calcium oxalate to carbonate or oxide, and titrated with hydrochloric acid. It appears possible, although not yet demonstrated in blood analyses, that the extra steps may increase the accuracy over that of the permanganate titration when the technique of Fiske and Adams for preparing the calcium oxide is followed. These authors wash the calcium oxalate with ammonium oxalate solution in a micro filter tube, then redissolve with dilute nitric acid and transfer to a platinum dish, where the solution is evaporated to dryness. The calcium nitrate residue is covered with 0.5 cc. of 2.5 per cent oxalic acid solution, is again dried, and finally ignited. The presence of oxalic acid causes the calcium oxide left by ignition to assume a loose form which is readily redissolved by 0.01 N HCl.

The CaO residue is dissolved in excess 0.01 N HCl and titrated back with 0.01 N NaOH. The Fiske and Adams technique makes it possible to substitute washing in a filter tube for washing by decantation and centrifugation, so that the possibility of the mechanical losses that can occur in the decantation are avoided.

Briggs (1) introduced quite another principle by precipitating the calcium as phosphate instead of oxalate and estimating the PO_4 colorimetrically. Roe and Kahn (13, 14) and Kuttner and Cohen (10) produced modifications of this method. It is less accurate than the permanganate titration when adequate amounts of material are present for the latter, but the colorimetric method has an advantage when minimal amounts must be determined. It can be used with a fraction of a cubic centimeter of plasma if necessary.

Blood cells contain little or no calcium (see the chapter on calcium, in volume I). Calcium determinations on whole blood are of little clinical value, because the calcium content of whole blood is determined less by the concentration of calcium in either cells or serum, than it is by the relative proportions of cells and serum in the blood.

Since calcium is precipitated by oxalates the latter can not be used as anticoagulants in blood the serum or plasma from which is to be used for calcium determination. If the whole blood is to be analyzed, 0.3 per cent potassium oxalate can be used for anticoagulant; when the proteins are precipitated with trichloroacetic acid the high acidity redissolves the calcium oxalate, so that it is all regained in the filtrate (2). However, whole blood analyses are seldom desirable. One usually centrifuges the cells and analyses the serum or plasma. And if oxalate has been added the calcium will be precipitated and mixed with the cells.

The serum or citrated plasma should be removed from the cells as soon as possible after the blood is drawn. If blood is allowed to stand for any considerable period the cell membranes appear to become permeable to calcium.

Choice of methods. For *urine*, descriptions will be given of McCrudden's gravimetric method, Shohl and Pedley's permanganate titration, Tisdall and Kramer's micro permanganate titration, and Van Slyke and Sendroy's gasometric method as applied by MacKay and Butler.

The amounts of urine required are, for the gravimetric method, 200 cc.; for Shohl and Pedley's titration, 100 cc.; for the micro titration, 2 cc.; for the gasometric method 3 to 10 cc. are ordinarily taken, but 1 cc. suffices.

In urine the reliable but laborious gravimetric method will seldom be resorted to, except as a standard when it seems desirable to check another procedure. Both the Shohl-Pedley titration method and the Van Slyke-Sendroy gasometric one are more rapid and convenient, and nearly as

accurate. The gasometric method has an advantage over the titration in that the gasometric can be used without preliminary oxidation of the urine to destroy uric acid.

If it is desirable to use but little urine the gasometric method is most convenient. The micro titration method of Tisdall and Kramer can also be used, but it must be preceded by the somewhat laborious Stolte dry ashing.

For *feces* the same methods are applied after the organic matter has been oxidized.

For *blood plasma or serum* micro permanganate titration, micro gasometric, and micro colorimetric methods are described. For the permanganate titration it is desirable to have the filtrate from as much as 2 cc. of serum; even the 0.2 mg. of Ca in this amount will require only 1.0 cc. of 0.01 N permanganate to titrate it. For the gasometric method filtrate from 1 cc. of serum yields an amount of calcium oxalate determinable with 1 per cent accuracy, and a fraction of a cubic centimeter can be used, with correspondingly less accuracy. The colorimetric method also permits determination on the filtrate from 1 cc. or less of serum, with accuracy more difficult to define.

CALCIUM DETERMINATION IN URINE

GRAVIMETRIC METHOD OF McCRUDDEN (11, 12)

Calcium is precipitated and isolated as the oxalate, the precipitate is ignited, and the calcium weighed as the oxide, CaO.

Reagents

2.5 per cent oxalic acid solution.
Twenty per cent sodium acetate solution.
0.5 per cent ammonium oxalate solution.

Procedure

The reaction of the urine is tested with litmus paper. If the urine is alkaline it is made neutral or slightly acid with hydrochloric acid solution. The neutral or slightly acid urine is then filtered; 200 cc. of the filtrate are transferred to an Erlenmeyer flask and the reaction is tested with litmus paper. If it is only faintly acid, 10 drops of concentrated hydrochloric acid are added; if it is strongly acid it is made just alkaline with strong ammonia water and then just acid with hydrochloric acid before the 10 drops of concentrated hydrochloric acid are

added. Ten cubic centimeters of 2.5 per cent oxalic acid are now introduced, followed by 8 cc. of 20 per cent sodium acetate. The mixture is either allowed to stand over night at room temperature or is shaken vigorously for ten minutes. It is then filtered through ash-free filter paper. The precipitate and flask are washed with 0.5 per cent ammonium oxalate solution until the precipitate is free from chloride. ***The filtrate and washings are used for the determination of magnesium by McCrudden's method*** (see chapter on magnesium).

The precipitate and filter paper are transferred to a platinum crucible, dried, ignited, and finally heated in a blast lamp to constant weight. The weight of the CaO is then determined.

Calculation

$0.7147\ W$ = grams of Ca in sample analyzed.

$$\frac{714.7\ W}{V} = \text{grams of Ca per liter of urine.}$$

$$\frac{35{,}680\ W}{V} = \text{milli-equivalents of Ca per liter of urine.}$$

W = grams of CaO weighed. V = cubic centimeters of urine represented in sample. One milli-equivalent of Ca = 20.03 mg.

Titration of calcium in urine by the method of Shohl and Pedley (17)

The urine is oxidized with ammonium persulfate to destroy excess uric acid, which would affect the permanganate titration. Calcium is precipitated as oxalate and the oxalate is determined by titration with permanganate.

Reagents

Concentrated sulfuric acid.

Concentrated ammonia.

Ammonium persulfate. This deteriorates when exposed to the air. The active salt, when first put into solution crackles, with the evolution of a gas that smells like ozone. It must be tested for the presence of calcium by a blank determination.

2.5 per cent oxalic acid solution.

0.02 per cent solution of methyl red in 50 per cent alcohol.

Sulfuric acid, approximately 1.0 N. Dilute 30 cc. of concentrated sulfuric acid to a liter.

Potassium permanganate, 0.05 N (for details of preparation and standardization see p. 31).

Procedure

To 100 cc. of unfiltered urine in a 250-cc. Erlenmeyer flask, add 5 cc. of concentrated sulfuric acid and 3 to 4 grams of ammonium persulfate. Insert a funnel into the neck of the flask to prevent the solution from spattering. Bring the liquid to a boil and keep it near the boiling point on an electric hot plate or over a low flame for one hour or until the persulfate is completely reduced and does not froth when the flask is agitated. At this point the solution should be pale green. Add 10 cc. of 2.5 per cent oxalic acid solution and cool the mixture to room temperature. Neutralize it with concentrated ammonia, using the methyl red indicator. Cool the solution to room temperature once more and if it becomes red bring it to the neutral point of the indicator again. Let it stand over night. Filter it on a 12.5 cm. hardened filter paper free from calcium. Wash the precipitate and the flask three times with water, filling the filter two-thirds full and allowing it to drain each time.

Break a hole in the filter paper and wash the precipitate back into the original flask, first with water and then with hot normal sulfuric acid, bringing the volume to about 100 cc. Add 10 cc. of concentrated sulfuric acid, heat the solution to 70 or 80°C. and titrate with 0.05 N potassium permanganate until the appearance of the first pink color that persists 15 seconds. A blank determination must be run on the reagents alone.

Calculation

$A - B$ = milligrams of Ca in sample.

$A - B$ = grams of Ca per liter of urine.

$50\ (A - B)$ = milli-equivalents of Ca per liter of urine.

A = cubic centimeters of 0.05 N $KMNO_4$ used in titrating sample. B = cubic centimeters used in titrating blank. V = cubic centimeters of urine represented in sample. To simplify the calculations of milligrams

and grams of Ca 20 instead of 20.03 is taken as the equivalent weight of Ca. The error introduced is negligible in this analysis.

MICRO TITRATION OF CALCIUM IN URINE BY THE METHOD OF TISDALL AND KRAMER (21)

Of the permanganate titration, a micro form, introduced by Halverson and Bergeim (5) for blood analysis, is applied to the redissolved ash of urine.

Reagents

A 10-fold dilution of ammonium hydroxide (10 cc. of concentrated ammonia solution diluted to 100 cc.).

A 50-fold dilution of ammonium hydroxide.

Sulfuric acid, approximately 1.0 N. Dilute 30 cc. of concentrated sulfuric acid to a liter.

Oxalic acid, 1.0 N. Dissolve 45 grams of oxalic acid in water and dilute to a liter.

A filtered saturated solution of sodium acetate.

Potassium permanganate, 0.01 N. This must be standardized each day against a 0.01 N solution of Sörensen sodium oxalate (for details of preparation and standardization see p. 31).

Procedure

The urine is ashed and extracted by the Stolte method described on page 70; the ash is dissolved, and the solution is brought to the original volume of the urine. Of the ash solution, 2 cc. are diluted to 4 cc. with water in a 15-cc. graduated centrifuge tube. (Centrifuge tubes used should be previously cleaned with chromic-sulfuric acid.) Add 1 drop of phenolsulfonephthalein (phenol red) indicator solution and introduce 10-fold diluted ammonium hydroxide solution drop by drop until the liquid is alkaline. Add 1.0 N sulfuric acid till it is just acid again to redissolve the phosphates. Add 1 cc. of 1.0 N oxalic acid followed by 1 cc. of a filtered saturated solution of sodium acetate. The latter must be added drop by drop. Mix and let the tube stand forty-five minutes. Centrifuge for ten minutes at 1300 revolutions per minute. Decant the supernatant fluid. Wash as described on page 770 for micro blood analyses. After the last washing fluid has been poured off add 2 cc. of 1.0 N sulfuric acid, shake the tube to suspend the

precipitate, warm it in a boiling water bath for a few minutes and titrate the solution with 0.01 N potassium permanganate until the first appearance of a pink color which persists one minute. A blank determination must be run on the reagents alone.

Calculation

$$0.2\,(A - B) = \text{milligrams of Ca in sample.}$$

$$\frac{0.2\,(A - B)}{V} = \text{grams of Ca per liter of urine.}$$

$$10\,(A - B) = \text{milli-equivalents of Ca per liter of urine.}$$

A = cubic centimeters of 0.01 N potassium permanganate used in the titration of the urine. B = cubic centimeters of 0.01 N potassium permanganate used in a blank determination on reagents. V = cubic centimeters of urine represented by the volume of urine extract.

GASOMETRIC DETERMINATION OF CALCIUM IN URINE

This method is described on page 425 in Chapter 7.

CALCIUM DETERMINATION IN FECES

GRAVIMETRIC METHOD OF McCRUDDEN (11, 12)

The procedure is similar in principle to that employed by McCrudden for analysis of urine.

Reagents

2.5 per cent oxalic acid solution.
Three per cent ammonium oxalate solution.
0.5 per cent ammonium oxalate solution.
Twenty per cent sodium acetate solution.
Concentrated ammonia solution.
0.1 per cent alizarin indicator solution.
Concentrated hydrochloric acid.

Procedure

Ashing. Stools may be ashed by any suitable procedure (see p. 69). Shohl and Rothwell (18), however, use the following especially for feces designed for calcium analyses.

The stool is treated with alcohol, dried, pulverized, and mixed by sieving, as described on page 78. A suitable portion[1] is weighed into a platinum crucible or dish and ashed slowly below red heat (about 400°C.) either in an electric oven or by the Stolte method (see p. 70). The ash is treated with 3 to 5 cc. of water and 1 cc. of concentrated nitric acid, and is heated until it is dissolved as entirely as possible. The solution is filtered through an ash free paper, crucible and paper being washed with 4 or 5 portions of about 5 cc. each of hot water.

For wet ashing the Neumann method (page 69) may be used.

Precipitation and ignition of calcium oxalate. The solution of ash is transferred to a 250-cc. Pyrex Erlenmeyer flask and diluted to about 100 cc. A few drops of alizarin solution are added, then concentrated ammonia drop by drop until the solution is just alkaline. Concentrated hydrochloric acid is then added until the solution is just acid. An additional 10 drops of the acid are then added, followed by 10 cc. of the 2.5 per cent oxalic acid.

From this point the procedure is the same as in the gravimetric determination of calcium in urine.

The filtrate and washings from the calcium oxalate may be used for determination of magnesium.

Calculation

$0.7147\ W$ = grams of Ca in the sample of stool analyzed.

$35.68\ W$ = milli-equivalents of Ca in the sample of stool analyzed.

W = weight of CaO in grams.

TITRATION OF CALCIUM IN FECES. McCRUDDEN (11, 12)

In this case the procedure up to the finish of the washing of the precipitate is the same as above described, except that a somewhat smaller sample, enough to yield about 20 to 30 mg. of Ca, is taken. The precipitate is washed 3 times with cold water, the filter being about two-

[1] Both amount and composition of feces vary greatly. The dry weight ordinarily varies from 25 to 75 grams per day, being highest in subjects on a vegetable diet. The calcium content varies with that of the food and with the intestinal behavior, but is likely to be 500 mg. or more per day. One may expect roughly about 1 to 2 per cent of the dry stool to be calcium. A suitable sample to yield 50 to 100 mg. of CaO by gravimetric analysis would therefore ordinarily be 0.1 to 0.2 of the twenty-four-hour feces, or about 5 grams of dried feces.

thirds filled each time and allowed to drain completely before more water is added. A hole is then made in the paper and the calcium oxalate is washed with 25 to 30 cc. of water into the same flask in which it was precipitated. The paper is then washed with 25 to 30 cc. of hot 1 N sulfuric acid to dissolve any oxalate crystals that may be adherent. To the washings 5 cc. of concentrated sulfuric acid are added and the oxalate is titrated immediately with 0.05 N potassium permanganate in the manner described in Shohl and Pedley's method for the analysis of urine, above.

$$(A - B) = \text{milligrams of Ca in sample.}$$

$$\frac{(A - B)}{20} = \text{milli-equivalents of Ca in sample.}$$

A = the number of cubic centimeters of permanganate used in the titration of the unknown solution, B = the cubic centimeters used in the titration of a blank determination on the reagents.

MICRO DETERMINATION OF CALCIUM IN FECES

A sample containing an amount of calcium suitable for one of the blood calcium methods is ashed, or a larger portion is ashed and aliquots with the proper amount of calcium for micro analysis are taken from the solution of the ash. The calcium is determined by one of the micro methods described below for blood analysis.

CALCIUM DETERMINATION IN BLOOD

MICRO TITRATION. MODIFIED METHOD OF HALVERSON AND BERGEIM (5)

The original picric acid method (5) of precipitating the proteins is replaced by the more convenient trichloroacetic acid technique used by Kramer and Tisdall (9). The calcium oxalate is precipitated in centrifuge tubes by an adaptation of McCrudden's principles. The precipitate is determined as in the original Halverson-Bergeim method, by redissolving in hot 1 N sulfuric acid and titrating the oxalic acid with 0.01 N permanganate.

Reagents

Trichloroacetic acid, 20 per cent. Twenty grams dissolved in water and diluted to 100 cc.

Sodium acetate, 20 per cent. Twenty grams of $NaC_2H_3O_2 \cdot 3H_2O$ dissolved and diluted to 100 cc.

Brom cresol green, 0.016 per cent. Stock 0.1 per cent solution is made as shown in table 60, page 793. Of the stock solution 16 cc. are diluted to 100.

1:1 Ammonia water. Concentrated ammonium hydroxide diluted with an equal volume of water.

1:50 Ammonia water. One volume of concentrated ammonia mixed with 50 volumes of water.

Ammonium oxalate, saturated solution, about 4 per cent.

Approximately 1 N sulfuric acid.

Potassium permanganate, 0.01 N. This can be made up the day used by dilution of standard 0.1 N permanganate, the preparation of which is described on page 31. An alternative procedure, used by Halverson and Bergeim, is to prepare a large volume of 0.01 N solution, and digest it at near the boiling point for thirty-six hours. The solution is then cooled and permitted to stand over night. The supernatant solution is syphoned from the precipitate of manganese dioxide which has settled to the bottom. Halverson and Bergeim state that after the solution has stood a few days it will usually lose not more than 0.1 per cent of its strength per week. It is standardized by titrating against the 0.01 N sodium oxalate solution as follows. Ten cubic centimeters of the oxalate solution and 10 cc. of 2 N sulfuric acid are heated to 65° to 70° and at once titrated with the permanganate until a color is obtained which persists for as long as a minute.

Sodium oxalate, 0.01 N. This is prepared by dilution of 0.1 N solution on the day used. For preparation of the 0.1 N solution see page 31.

Removal of proteins

Serum or citrate plasma is pipetted into a measuring flask calibrated to hold five times the volume of the sample. Three volumes of water are mixed with the sample, and then one volume of freshly prepared 20 per cent trichloroacetic acid, filling the flask up to the mark. The material is mixed and allowed to stand a half hour for precipitation of the proteins. The mixture is then transferred to a tube and centrifuged. The supernatant liquid is poured off through a small ashless filter paper and filtrate is obtained of about four-fifths the volume of the mixture. When sufficient material is available 5 cc. of serum or plasma are taken, and yield enough filtrate for duplicate analyses.

Rothwell (15) has shown that the same procedure can be applied to whole blood containing as much as 0.3 per cent of potassium oxalate. The blood is precipitated with 7 volumes of water and 2 volumes of 20 per cent of trichloroacetic acid, or is mixed directly with 9 volumes of 5

per cent trichloroacetic acid. In oxalate blood the calcium is already precipitated, but the high acidity of the trichloroacetic acid apparently completely redissolves the calcium oxalate.

In case minimal amounts of material must be used 1 cc. of blood or serum can be ashed, the ash redissolved in dilute hydrochloric or nitric acid, and the entire redissolved ash used for analysis as described below (7).

Precipitation of calcium oxalate

Filtrate equivalent to 0.5 or 1.0 cc. of serum suffices when the precipitated oxalate is to be determined gasometrically (p. 421) or by the colorimetric method (p. 773). For titration, however, one preferably uses at least 10 cc. of serum filtrate, equivalent to 2 cc. of serum, or 15 to 20 cc. of blood filtrate, equivalent to 3 or 4 cc. of whole blood. These amounts will contain about 0.2 mg. of Ca, and require about 1 cc. of 0.01 N permanganate in the titration. If smaller amounts are taken the titration error of about ± 0.01 cc. of the permanganate becomes more significant, and the effects of any errors in the washing of the calcium oxalate are magnified. If the sample represents only 1 cc. of serum a possible error of ± 5 per cent must be expected.

In a scrupulously clean[2] 15 cc. graduated conical centrifuge tube are placed 10 cc. of filtrate, or a smaller volume of filtrate which is diluted to 10 cc. in the tube. (In case filtrate from whole blood is used, 20 cc. are concentrated to 10 cc.) One cubic centimeter of 20 per cent sodium acetate, 6 or 8 drops of 0.016 per cent brom cresol green indicator, and 1 cc. of saturated ammonium oxalate are added. In adding the oxalate care is taken that it drops directly into the solution and does not touch the lip of the tube, from which removal by the subsequent washing is likely to be incomplete. The mixture is stirred with a thin-footed, glass rod. A few drops of 1:1 ammonia are added until the resulting color matches that of a similar volume of acetate buffer solution of pH 5.0, containing the same number of drops of indicator. Shohl (16) has shown that at pH above 4.0 calcium oxalate is completely precipitated. The stirring rod is washed off with a few drops of water. The tube is covered and the mixture is allowed to stand over night to complete precipitation. (For acetate buffer, see table 69, p. 813.)

[2] The tubes may either be kept immersed in a mixture of chromate and sulfuric acid whenever not in use (23), or may be heated at about 100° in the mixture immediately before they are used (3).

Washing the calcium oxalate precipitate

The washing of the calcium oxalate by centrifugation and decantation is the point at which, workers who have studied the method agree (3, 19, 23), there is most possibility of error. In American laboratories the washing has been done with distilled water, as in Halverson and Bergeim's original procedure, or with dilute ammonia. Small volumes of washing fluid have been used, so that slight losses of the precipitated calcium oxalate, by its resolution or mechanical escape in the washings, are balanced by retention of equivalent amounts of ammonium oxalate not completely removed by the washings. In England, Stanford and Wheatley (19) have introduced the use of saturated calcium oxalate solution as washing fluid, so that loss by resolution of the precipitate is avoided.

We shall describe three washing techniques, each of which has proven its practicability in routine analysis. The choice among them will depend upon the conditions of the analyses.

1. Clark and Collip's washing method by decantation. (3). After centrifugation of the calcium oxalate is complete the supernatant liquid is carefully poured off. While the tube is still inverted it is placed in a rack for five minutes to drain, the mouth of the tube resting on a pad of filter paper. The mouth of the tube is wiped dry with a soft cloth and the sides of the tube are washed down with 3 cc. of 1:50 ammonia solution directed in a very fine stream from a wash bottle. The precipitate is stirred up with the 3 cc. of washing fluid, and is again centrifuged. The supernatant solution is decanted and the tube is drained for five minutes as before. The precipitate is then ready to be redissolved in sulfuric acid and titrated.

The Clark and Collip washing technique can be used only with conical centrifuge tubes, which are sufficiently narrow at the bottom (about 3 mm. inner diameter) to hold together the precipitate and permit none to escape when the fluid is decanted.

After the five minutes' drainage only about 0.02 cc. of solution remains adherent to the walls of the tube. Consequently one can calculate that after the single washing there remains in the tube, from the 35 mg. of ammonium oxalate added to precipitate the calcium, only about 0.0005 mg., equivalent to 0.0002 mg. of Ca, or 0.1 per cent of the amount usually determined in a 2 cc. sample of plasma. This is negligible. The amount of error caused in the opposite direction by solubility of the calcium oxalate in the 3 cc. of washing fluid also is negligible. The

slight amount of ammonium oxalate, about 0.01 mg., left after the firs decantation, is sufficient to depress the solubility of the calcium oxalatе so far that the theoretical maximum loss from solubility would be onl a fraction of a per cent of the calcium present, and the actual loss ir the short period of contact must be still less.

Of the three methods of washing which we describe, the Clark anc Collip is the simplest with regard to manipulation, and involves onl a single washing. Its only drawback is the occurrence of occasional loss probably from unnoticed agitation of the precipitate during the decantations. When there is sufficient material to repeat an occasional duplicate that goes wrong from this cause, the method is the most convenient available.

2. ***The Halverson-Bergeim washing with removal of mother liquors by suction*** (5, 23). The procedure is here described as somewhat modified by Van Slyke and Sendroy (23). After precipitation of the calcium oxalate is complete the tube is centrifuged, and the supernatant solution is carefully and slowly drawn off without disturbing the precipitate. For drawing off the fluid a piece of thin walled glass tubing is drawn out to a capillary which is bent back into a U, the returning limb of which is cut off at a height 1 or 2 mm. above the bend. Suction is applied to this tube through a rubber tube which can be opened and closed by a pinch cock attached near the point where the rubber joins the glass. The glass capillary with the bent tip is immersed into the solution above the calcium oxalate, and the suction is permitted to act by opening slightly the pinck-cock. The solution is drawn off until only 0.2 or 0.3 cc. remains above the precipitate. Some calcium oxalate crystals are likely to remain in the surface film of the liquid even after centrifuging. To avoid loss of these, one takes care that during removal of the solution the curved tip of the tube is kept ***always below the surface,*** that the suction is applied so slowly that the solution is not agitated, and that the suction is stopped before the surface of the fluid has fallen to the inlet of the suction tube.

The precipitate and tube are washed with two portions of the 1:50 ammonia water. In each washing 3 cc. of the solution are poured gently down the walls of the tube so that the latter are washed about their entire circumference ***with least possible disturbance of the precipitate.*** The mixture is then centrifuged and the liquid is drawn off as above outlined.

If at each withdrawal 0.25 cc. of fluid is left in the tube one can calcu-

late that after the second washing the amount of ammonium oxalate left is equivalent to about 0.002 mg. of calcium, or 1 per cent of the amount ordinarily in 2 cc. of serum. This retention is balanced by a slight loss of precipitate, so that the results are exact within the limit of error of the titration (23).

The above procedure requires more time and manipulation than the Clark-Collip technique, and involves two washings instead of one. It appears, however, to be more immune to mechanical losses of the precipitate, and is favored when the repetition of an occasional analysis is undesirable.

3. Stanford and Wheatley's (19) washing method. A saturated calcium oxalate solution is prepared by shaking pure, thoroughly washed precipitated calcium oxalate with water at room temperature. The solution is filtered through two thicknesses of No. 30 Whatman paper to remove fine crystals. In order to be sure that the removal has been complete 15 cc. of the solution plus 1 cc. of concentrated sulfuric is titrated, as described below, with 0.01 N permanganate. From the solubility of calcium oxalate, enough should be dissolved so that the titration takes 0.17 cc. of 0.01 N permanganate. The solution is satisfactory if it does not take more than 0.25 cc.

After precipitation and centrifugation of the calcium oxalate in the serum filtrate, the supernatant fluid is sucked off with a bent capillary, and is washed twice with a volume of the saturated calcium oxalate solution about equal to the volume of fluid (10 cc.) in which the calcium was precipitated. The technique of washing and of withdrawing the fluid with a bent capillary tube is the same described above for the Halverson-Bergeim procedure.

The Stanford and Wheatley method appears to be the safest of all, since the danger either of mechanical loss by decantation or of loss by resolution of the precipitate is avoided. Also such large volumes of washing fluid can be used that the fluid need not be drawn off from the precipitate at each washing as completely as in the Halverson-Bergeim procedure. When 10 cc. of washing fluid are used, even if 0.5 cc. is left after each removal no significant error is caused. The Stanford-Wheatley method would seem to be especially desirable when the smallest amounts of calcium are determined, as in gasometric micro analyses on 1.0 or 0.5 cc. samples of serum. The only disadvantage of the Stanford-Wheatley method is the necessity of preparing an extra reagent, the saturated calcium oxalate.

Titration of the calcium oxalate precipitate

Two cubic centimeters of the approximately 1 N sulfuric acid are blown from a pipette directly onto the precipitate in such a manner as to break it up and facilitate its solution. The tube is then placed in a boiling water bath for about one minute. It is then placed in a beaker containing water heated to 70° to 75°, and is there titrated with 0.01 N permanganate from a micro burette graduated into 0.02 cc. divisions (see figure 1, p. 13), until the appearance of a definite pink color which persists 1 minute. The error of the titration is only about ±0.01 cc. Variations in results not accounted for by a ±0.01 cc. error in titration are likely to be attributable to the washing technique.

Calculation

$$0.2\,f\,(A - B) = \text{milligrams of Ca in sample.}$$

$$\frac{20\,f\,(A - B)}{V} = \text{milligrams of Ca per 100 cubic centimeters of serum.}$$

$$\frac{10\,f\,(A - B)}{V} = \text{milli-equivalents of Ca per liter of serum.}$$

A indicates the cubic centimeters of 0.01 N permanganate used in the titration, B the cubic centimeters used in titration of a blank, f the 0.01 N factor of the permanganate obtained by titration against 0.01 N sodium oxalate (f is the ratio, cubic centimeters of oxalate: cubic centimeters of 0.01 N permanganate obtained in the standardization). V is the cubic centimeters of blood or plasma represented in the portion of filtrate analyzed.

COLORIMETRIC BLOOD CALCIUM. BRIGGS METHOD (1) AS MODIFIED BY ROE AND KAHN (14)

The calcium is precipitated as phosphate and estimated by colorimetric determination of the PO_4 by the methods described in the phosphorus chapter.

Reagents

Standard phosphate solution. (a) *Stock solution.* Dissolve 2.265 grams of pure dry monopotassium phosphate in 1 liter of phosphate-free water. One cubic centimeter of this reagent contains 0.5162 mg. of phosphorus equivalent to 1 mg. of calcium as $Ca_3(PO_4)_2$. Preserve with chloroform.

(b) *Phosphate solution for calcium estimation.* Pipette accurately 20 cc. of the stock phosphate solution into a liter flask and make up to the mark

with phosphate-free water. Five cubic centimeters of this solution contain 0.05162 mg. of phosphorus, equivalent to 0.1 mg. of calcium as $Ca_3(PO_4)_2$.

Alkaline alcohol wash reagent. In a 100-cc. graduated cylinder place 58 cc. of 95 per cent ethyl alcohol. Add 10 cc. of amyl alcohol and make up to 100 cc. with distilled water. Add 2 drops of 1 per cent phenolphthalein. Now add 5 per cent calcium-free sodium hydroxide, a drop at a time, with repeated shaking, until a distinct pink is obtained. Two or 3 drops of the alkali are usually enough to alkalinize properly a mixture of neutral alcohols.

Procedure

To 4 parts of 10 per cent trichloroacetic acid in an Erlenmeyer flask add 1 part of blood serum. Shake the flask until the contents are thoroughly mixed. Filter through a calcium-free filter paper (Whatman No. 42). Place 5 cc. of the filtrate in a 15-cc. conical graduated centrifuge tube, add 1 cc. of 25 per cent calcium-free sodium hydroxide, and let stand for five minutes. Add 1 cc. of 5 per cent trisodium phosphate, twirl the tube until the contents are thoroughly mixed, and set aside for one hour.

After one hour's standing centrifuge for two minutes. Decant the supernatant liquid and place the tube in an inverted position in a small beaker containing a mat of clean gauze or filter paper in the bottom. Allow to drain for two minutes, then wipe the mouth of the tube dry with a clean cloth. Add from a pipette 5 cc. of the alkaline alcoholic wash reagent, delivering the reagent in a manner that will first break up the mat of $Ca_3(PO_4)_2$ in the bottom of the tube and then wash down the sides of the tube. This is done by using a bulb pipette with a fine delivery tip. The contents of the pipette are first blown forcefully by means of the breath upon the mat of $Ca_3(PO_4)_2$ and are later directed upon the sides of the tube. If the mat of $Ca_3(PO_4)_2$ is not broken up completely by this procedure, it must be fragmented thoroughly with a clean glass stirring rod. Centrifuge for two minutes, then decant the wash reagent. Drain the tube as indicated above by placing in an inverted position for two minutes and wipe the mouth dry with a clean cloth.

Redissolve the calcium phosphate in 5 cc. of 5 per cent trichloroacetic acid, and analyze by any of the methods described in the phosphorus chapter for colorimetric determination of inorganic phosphate in serum or plasma (Tisdall's method is preferred by the authors). For the colorimetric comparison, five cubic centimeters of standard

phosphate solution *b*, containing phosphate equivalent to 0.1 mg. of calcium, are used.

Calculation

When the sample represents 1 cc. of serum, the standard is equivalent to 0.1 mg. of Ca, and both standard and unknown are brought to the same volume for color comparison, the following formulae are used:

$$\frac{10\ S}{U} = \text{milligrams of Ca per 100 cubic centimeters of serum.}$$

$$\frac{5\ S}{U} = \text{milli-equivalents of Ca per liter of serum.}$$

S = reading of standard, U = reading of unknown.

When the standard is other than equivalent to 0.1 mg. of Ca, or its volume differs from that of the unknown, the results calculated by the above formulae are multiplied by the factor:

$$\frac{\text{mg. Ca in standard}}{0.1} \times \frac{\text{volume of unknown}}{\text{volume of standard}}$$

COLORIMETRIC ULTRA MICRO METHOD OF KUTTNER AND COHEN FOR BLOOD AND PUS

Kuttner and Cohen (10) by a similar procedure determine the Ca in as little as 0.1 cc. of plasma or pus. In order to avoid the loss which attends precipitating the proteins, they ash the material in platinum, first by dry heat, then with a drop or two of concentrated nitric acid. The ash is dissolved in 7 per cent trichloroacetic acid, and the rest of the procedure is similar to that above outlined, except that everything is done with smaller quantities. The $Ca_3(PO_4)_2$ is precipitated in a small tube from 1 cc. of solution, and is washed twice with 1 cc. portions of washing solution. The colorimetric determination is done in small tubes by diluting the unknown until it matches the standard.

GASOMETRIC BLOOD CALCIUM

This method is described on page 421 of chapter VII.

BIBLIOGRAPHY

1. Briggs, A. P.: Some applications of the colorimetric phosphate method. *J. Biol. Chem.*, 1924, 59, 255.

2. Clark, G. W.: The micro determination of calcium in whole blood, plasma, and serum by direct precipitation. *J. Biol. Chem.*, 1921, **49**, 487.
3. Clark, E. P., and Collip, J. B.: A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. *J. Biol. Chem.*, 1925, **63**, 461.
4. Corley, R. C., and Denis, W.: The determination of calcium in tissues, feces, and milk. *J. Biol. Chem.*, 1925, **66**, 601.
4a. Fiske, C. H., and Adams, E. T.: Determination of calcium by alkalimetric titration. *J. Am. Chem. Soc.*, 1931, **53**, 2498.
5. Halverson, J. O., and Bergeim, O.: The determination of small amounts of calcium, particularly in blood. *J. Biol. Chem.*, 1917, **32**, 159.
6. Hamilton, B.: A comparison of the concentrations of inorganic substances in serum and spinal fluid. *J. Biol. Chem.*, 1925, **65**, 101.
7. Kramer, B., and Howland, J.: Method for determination of calcium in small quantities of blood serum. *J. Biol. Chem.*, 1920, **43**, 35.
8. Kramer, B., and Tisdall, F. F.: A simple technique for the determination of calcium and magnesium in small amounts of serum. *J. Biol. Chem.*, 1921, **47**, 475.
9. Kramer, B., and Tisdall, F. F.: The direct quantitative determination of sodium, potassium, calcium, and magnesium in small amounts of blood. *J. Biol. Chem.*, 1921, **48**, 223.
10. Kuttner, T., and Cohen, H. R.: Micro colorimetric studies. I. A molybdic acid, stannous chloride reagent. The micro estimation of phosphate and calcium in pus, plasma, and spinal fluid. *J. Biol. Chem.*, 1927, **75**, 517.
11. McCrudden, F. H.: The quantitative separation of calcium and magnesium in the presence of phosphates and small amounts of iron, devised especially for the analysis of foods, urine and feces. *J. Biol. Chem.*, 1909–10, **7**, 83.
12. McCrudden, F. H.: The determination of calcium in the presence of magnesium and phosphates: the determination of calcium in urine. *J. Biol. Chem.*, 1911–12, **10**, 187.
13. Roe, J. H., and Kahn, B. S.: A colorimetric method for the estimation of blood calcium. *J. Biol. Chem.*, 1926, **67**, 585.
14. Roe, J. H., and Kahn, B. S.: The colorimetric determination of blood calcium. *J. Biol. Chem.*, 1929, **81**, 1.
15. Rothwell, C. S.: The determination of calcium in whole oxalated blood. *J. Biol. Chem.*, 1927, **74**, 257.
16. Shohl, A. T.: The effect of hydrogen ion concentration upon the determination of calcium. *J. Biol. Chem.*, 1922, **50**, 527.
17. Shohl, A. T., and Pedley, F. G.: A rapid and accurate method for calcium in urine. *J. Biol. Chem.*, 1922, **50**, 537.
18. Shohl, A. T., and Rothwell, C. S.: Personal communication.
19. Stanford, R. V., and Wheatley, A. H. M.: The estimation of calcium in blood. *Biochem. J.*, 1925, **19**, 710.
20. Tisdall, F. F.: A note on the Kramer-Tisdall method for the determination of calcium in small amounts of serum. *J. Biol. Chem.*, 1923, **56**, 439.
21. Tisdall, F. F., and Kramer, B.: Methods for the direct quantitative determination of sodium, potassium, calcium and magnesium in urine and stools. *J. Biol. Chem.*, 1921, **48**, 1.

22. TREVAN, J. W., AND BAINBRIDGE, H. W.: Estimation of calcium in blood serum. *Biochem. J.*, 1926, **20**, 423.
23. VAN SLYKE, D. D., AND SENDROY, J., JR.: Gasometric determination of oxalic acid and calcium, and its application to serum analysis. *J. Biol. Chem.*, 1929, **84**, 217.

CHAPTER XXVI

Magnesium

DISCUSSION

Earlier methods for the estimation of magnesium in biological material involved ashing of the material, removal of the calcium as oxalate, precipitation of the magnesium as $MgNH_4PO_4 \cdot 6H_2O$, and ignition of the precipitate to pyrophosphate, $Mg_2P_2O_7$, which was weighed. McCrudden (14, 15) simplified the method for urine by employing a procedure which obviated ashing. Jones (9) showed that ignition may be omitted, and that the $MgNH_4PO_4 \cdot 6H_2O$ crystals can be dried to constant weight with the designated content of water of crystallization in sixteen hours or longer at room temperature, at least in atmosphere of the humidity of Baltimore. Most chemists still ignite to the pyrophosphate, however. The gravimetric method is simple and exact, and a considerable number of analyses can be run through in a day, particularly if Jones' method can be used, or if a muffle furnace is available so that a number of crucibles can be ignited together.

A titration method based on the magnesium ammonium phosphate precipitate was devised in 1877 by Stolba (16). This tribasic alkaline phosphate was titrated to the monobasic by means of acid according to the reaction:

$$B_3PO_4 + 2\ HCl = BH_2PO_4 + 2\ BCl,$$

with cochineal as indicator to show the end point, which is at pH 4.5 to 4.7. Tisdall and Kramer (17) have applied the method to the determination of magnesium in urine.

For clinical blood analyses the above procedures require too much material (unless a micro balance is used) and colorimetric methods must be employed. These (1, 2, 3, 4, 7, 11, 12, 13) all depend upon colorimetric determination of the PO_4 in the magnesium ammonium phosphate precipitate, and have utilized in turn the various colorimetric micro methods that have been developed for phosphate. They are not so exact as the classical gravimetric procedure or the titration, which are preferable for analysis of urine and stools, where sufficient material is available. Resort to the colorimetric micro methods is desirable only for blood.

Among other methods proposed for the analysis of blood and serum is the micro-gravimetric procedure of Dienes (5), which utilizes Pregl's micro-technique. Kolthoff (10) has suggested the use of "Titan-yellow" for colorimetric determination of magnesium.

The amount of magnesium in the normal excreta varies with that in the diet, but is usually 100 to 200 mg. per twenty-four hours in the urine, and two or three times as much in the feces. In normal serum the magnesium content is 1 to 3 mg. per 100 cc. or 1 to 2.5 univalent milli-equivalents per liter (see volume I).

MAGNESIUM IN URINE

Gravimetric method of McCrudden (14, 15)

Reagents

Concentrated nitric acid.

Concentrated hydrochloric acid.

Dilute ammonia solution (specific gravity about 0.96), 1 volume of concentrated ammonia diluted with 2 volumes of water.

Two per cent acid sodium phosphate solution.

0.1 per cent alizarine solution.

Alcoholic ammonia solution, 1 part of alcohol, 1 part of dilute ammonia (specific gravity 0.96) and 3 parts of water.

Procedure

Calcium is precipitated from the urine by the method of McCrudden (see calcium determination, p. 761). The calcium-free filtrate secured by removal of the calcium oxalate is transferred to a porcelain dish; 20 cc. of concentrated nitric acid are added, and the mixture is evaporated to dryness. The residue is heated over a free flame until the ammonium salts are destroyed and fumes of nitric oxide no longer come off. Then 10 cc. of concentrated hydrochloric acid are added and the solution is again evaporated nearly to dryness. It is then diluted to about 80 cc., nearly neutralized with ammonia and then cooled. Enough acid sodium phosphate solution (usually 10 or 20 cc.) is added to precipitate the magnesium. With a few drops of alizarine as indicator, ammonia is then added drop by drop with constant stirring until the solution is alkaline, and then in addition enough dilute ammonia to increase the total volume of the solution by one-fourth. The mixture is allowed to stand over night. Next day it is filtered on a Gooch or porous-bottomed Jena glass crucible of coarse porosity. The precipitate is washed free of chlorides

with alcoholic ammonia solution. The crucible is dried, and ignited at a bright red to change the precipitate to $Mg_2P_2O_7$.

An alternative procedure to ignition is to let the crucible dry in room air over night or longer, until the weight is constant, and then weigh the precipitate as $MgNH_4PO_4 \cdot 6H_2O$, according to Jones (9). The writers have not had experience with this procedure, but it appears that it may be advantageous when numbers of analyses are to be done and a muffle furnace is not available.

Calculation

When the precipitate is weighed as $Mg_2P_2O_7$:

$0.2184\ W$ = grams of Mg in sample.

$17.96\ W$ = milli-equivalents of Mg in sample.

$\frac{218.4\ W}{V}$ = grams of Mg per liter of urine.

$\frac{17{,}960\ W}{V}$ = milli-equivalents of Mg per liter of urine.

When the precipitate is weighed as $MgNH_4PO_4 \cdot 6H_2O$:

$0.09906\ W$ = grams of Mg in sample.

$8.15\ W$ = milli-equivalents of Mg in sample.

$\frac{99.06\ W}{V}$ = grams of Mg per liter of urine.

$8{,}146\ W$ milli-equivalents of Mg per liter of urine.

W represents grams of precipitate and V the cubic centimeters of urine in the sample analyzed. One milli-equivalent of Mg is calculated as

$$\frac{24.32}{2} = 12.16 \text{ milligrams of magnesium.}$$

MAGNESIUM IN URINE. TITRATIOM METHOD OF TISDALL AND KRAMER (17)

The determination is based on the precipitation of magnesium as magnesium ammonium phosphate and titration of the tertiary to the primary phosphate with an indicator which gives the correct end point (about pH 4.6) for completion of the reaction. The latter may be represented as

$$Mg(NH_4)PO_4 + 2\ HCl = MgCl_2 + H_2(NH_4)PO_4.$$

In practice the precipitate is dissolved with an excess of acid, which is titrated back with alkali.

Reagents

A 10 volume per cent solution of ammonium hydroxide. Dilute 10 cc. of concentrated ammonia solution to 100 cc.

An approximately 4 N sulfuric acid solution. Dilute 110 cc. of concentrated sulfuric acid to a liter.

A saturated solution of ammonium oxalate.

A 10 per cent solution of ammonium phosphate $(NH_4)_2HPO_4$.

Thirty volume per cent alcohol. Dilute 30 cc. of alcohol to 100 cc.

Tincture of cochineal. Extract 1 part of crushed cochineal with 10 parts of 25 volume per cent alcohol.

0.1 N hydrochloric acid solution.

0.1 N sodium hydroxide solution.

Procedure

Twenty-five to 50 cc. of urine are dried and are ashed by the method of Stolte, described on page 70 of chapter II. To the redissolved ash in a 100-cc. beaker add a drop of phenol red indicator and enough 10 per cent ammonium hydroxide to make the solution just alkaline. Add 4 N sulfuric acid till it is just acid again to redissolve the phosphates. Introduce 5 cc. of saturated ammonium oxalate, mix, and let the mixture stand 15 minutes to precipitate the calcium. To insure the presence of an excess of phosphate add 1 cc. of 10 per cent ammonium phosphate, followed by 5 cc. of concentrated ammonia water. Mix the contents of the beaker thoroughly and, after the mixture has stood an hour,[1] filter it through a 9-cm. No. 40 Whatman filter paper, transfering the whole precipitate from the beaker to the filter with the aid of a rubber-tipped rod and some 10 per cent ammonium hydroxide. Remove all the ammonia from the filter by washing the latter four times with 30 per cent alcohol. Transfer the

[1] Up to this point McCrudden's procedure, described above, for precipitation of the $MgNH_4PO_4$ may be used instead of the Tisdall-Kramer method. The McCrudden precipitation is carried out with larger amounts of urine, so that 20 to 40 cc. of 0.1 N HCl may be required to redissolve the precipitate instead of the 5 cc. which usually suffice when the Tisdall-Kramer directions are followed. The McCrudden method avoids the Stolte ashing and the consequent necessity of platinum dishes. When the McCrudden precipitation is used the amount of indicator added is increased in proportion to the volume of solution that is present during the titration.

filter paper with the precipitate to a 100-cc. beaker, add about 30 cc. of warm water and stir filter paper and precipitate into the water with a glass rod. Add 3 drops of tincture of cochineal and an excess (usually 5 cc.) of 0.1 N hydrochloric acid. After five minutes titrate the solution with 0.1 N sodium hydroxide until the light yellow color changes to purple. Use a burette graduated into 0.05 cc. divisions.

The accuracy of the end point may be increased by Fiske's (6) device of using for comparison a flask containing a solution of acetate buffer at the pH of the desired end point. The buffer solution is made by adding 50 cc. of 2 N acetic acid to 35 cc. of 2 N sodium hydroxide solution (the acid and hydroxide should titrate exactly against each other with phenolphthalein as indicator). This stock buffer solution is diluted 10-fold for use. The control flask contains a volume of the diluted buffer solution approximately equal to the final volume of solution in the titrated flask, and an equal amount of indicator is added to each. Fiske prefers as indicator methyl red solution (see table 68, p. 812), added in sufficient amount to make one-tenth the volume of the final solution. Bromcresol green should also be applicable (table 68).

Calculation

$$1.216\ (A - B) = \text{milligrams of Mg in sample.}$$

$$\frac{1.216\ (A - B)}{V} \quad \text{grams of Mg per liter of urine.}$$

$$\frac{100\ (A - B)}{V} = \text{milli-equivalents of Mg per liter of urine.}$$

A = cubic centimeters of 0.1 N HCl; B = cubic centimeters of 0.1 N NaOH; V = cubic centimeters of urine in sample.

MAGNESIUM IN URINE. COLORIMETRIC METHOD OF BRIGGS (2)

Brigg's method depends on the colorimetric determination of the phosphorus in precipitated magnesium ammonium phosphate. This is less accurate than titration, but can be performed with very little urine.

Reagents

Reagents for colorimetric phosphate determination (see chapter XXXI).

Dilute ammonia solution. Dilute 25 cc. of concentrated ammonia water to 100 cc.

Five volumes per cent solution of glacial acetic acid.

Four per cent solution of ammonium oxalate.

Two per cent solution of potassium dihydrogen phosphate.

Alcohol wash solution. Dilute 200 cc. of 95 per cent alcohol and 50 cc. of concentrated ammonia water to a liter.

An approximately 0.2 N hydrochloric acid solution. Dilute 16 cc. of concentrated hydrochloric acid to a liter.

Methyl red, 0.05 per cent solution in alcohol.

Procedure

To 2 cc. of clear, acid urine in a Pyrex test tube, 16 by 150 mm., marked to contain 15 cc., add a drop of methyl red indicator and introduce dilute ammonia, drop by drop, until the color of the solution changes to brown. Adjust the reaction with a few drops of 5 per cent acetic acid if too much ammonia is added. Add 1 cc. of 4 per cent ammonium oxalate and rub down the calcium oxalate precipitate with a rubber-tipped stirring rod. Set the tube aside for two hours. Add 1 cc. of 2 per cent potassium phosphate and 1 cc. of concentrated ammonia water and rub down the precipitate of magnesium ammonium phosphate. Allow the mixture to stand two hours. Centrifuge ten minutes at 1500 r.p.m. and pour off the supernatant fluid. Add 20 cc. of the alcoholic wash solution, rub the sides of the tube and stir up the precipitate with the rubber tipped rod. Centrifuge again. Repeat the washing. Dissolve the precipitate, after decantation of the final supernatant fluid, in a small amount of 0.2 N hydrochloride acid. Determine the phosphorus by either of the colorimetric methods described in the phosphorus chapter, using for comparison a standard phosphate solution containing 0.10 mg. of phosphorus.

Calculation

$$\frac{0.0784}{V} \times \frac{S}{U} = \text{grams of Mg per liter of urine.}$$

$$\frac{6.45}{V} \times \frac{S}{U} = \text{milli-equivalents of Mg per liter of urine.}$$

S and U represent readings of standard and unknown respectively. V is the number of cubic centimeters of urine taken for analysis.

MAGNESIUM IN FECES. GRAVIMETRIC METHOD OF McCRUDDEN (14, 15)

Reagents

Concentrated nitric acid.

Concentrated hydrochloric acid.

Dilute hydrochloric acid, about 0.5 N

Dilute ammonia solution (specific gravity about 0.96), 1 volume of concentrated ammonia solution diluted with 2 volumes of water.

Two per cent acid sodium phosphate solution.

Five per cent sodium citrate solution.

0.1 per cent alizarin red solution.

Alcoholic ammonia solution. One part of alcohol, 1 part of dilute ammonia solution (specific gravity 0.96) and 3 parts of water.

Procedure

For the determination of magnesium the calcium-free filtrate, obtained in the McCrudden calcium method (see p. 765) after the calcium oxalate has been removed, is used. It is treated in the same manner as the urine filtrate (see above) except that the presence of iron, which is frequently found in stools, necessitates treatment with sodium citrate.

The filtrate containing the magnesium is evaporated almost to dryness in a porcelain dish, after the addition of 20 cc. of concentrated nitric acid. Heating is continued until fumes of nitric oxide no longer come off. Ten cubic centimeters of concentrated hydrochloric acid are added and the solution is again evaporated nearly to dryness. It is then diluted to about 80 cc., nearly neutralized with ammonia, and then cooled.

If no iron is present the procedure described for urine analysis may be continued from this point on. If iron is present 0.5 to 1.0 cc. of 5 per cent sodium citrate is added, followed by enough acid sodium phosphate to precipitate the magnesium. A few drops of 1 per cent alizarine red solution are introduced, and dilute ammonia solution is added drop by drop, with constant stirring, until the solution is alkaline. Enough additional ammonia is then introduced to inrease the volume of the solution by one-fourth. The solution is allowed to stand over night. Next day it is filtered and washed a few times by decantation with the alcoholic ammonia solution. The precipitate on the filter paper and in the flask is then dissolved in dilute hydrochloric acid and made up to 80 cc. with water. Then 0.5 to 1.0 cc. of 5 per cent sodium citrate is added, the magnesium is reprecipitated with sodium acid phosphate and ammonia solution and filtered, as before. The precipitate is collected in a Gooch or porous-bottomed Jena glass crucible, as described above for urine analysis, and is weighed either as air-dried $MgNH_4PO_4 \cdot 6H_2O$ or as ignited $Mg_2P_2O_7$.

Calculation of magnesium in sample is same as for urine.

MAGNESIUM IN FECES. TITRATION METHOD OF TISDALL AND KRAMER (17)

Like Tisdall and Kramer's titrimetric method for urine, this is an application of Stolba's alkalimetric titration of the $MgNH_4PO_4$ precipitate.

Of a solution of redissolved stool ash prepared by the Tisdall-Kramer technique described on page 70 of chapter II, 10 to 30 cc. are analyzed by the same procedure described for the analysis of urine.

Calculation

$$1.216\ (A - B) = \text{milligrams of Mg in sample analyzed.}$$

$$0.1\ (A - B) = \text{milli-equivalents of Mg in sample analyzed.}$$

A = cubic centimeters of 0.1 N HCl, B = cubic centimeters of 0.1 N NaOH.

As in urine analysis, the titration procedure may be applied in place of weighing to the magnesium ammonium phosphate precipitate obtained in the McCrudden method. The calculation is the same as in the Tisdall-Kramer method.

MAGNESIUM IN SERUM. COLORIMETRIC METHOD OF BRIGGS (2)

The magnesium is precipitated as $MgNH_4PO_4 \cdot 6H_2O$, and the PO_4 is determined colorimetrically.

Reagents

The reagents for Halverson and Bergeim's calcium method described on page 767 of the preceding chapter.

The reagents for whichever colorimetric phosphorus method is chosen (see pages 875–880 of the phosphorus chapter).

In addition the following:

A 2 per cent solution of KH_2PO_4.

Concentrated ammonia solution.

Ammoniacal dilute alcohol, 200 cc. of 95 per cent alcohol and 50 cc. of concentrated ammonia solution plus water to 1 liter.

Standard KH_2PO_4 *solution* containing 0.025 mg. of P per cubic centimeter. The solution contains 0.1098 gram of KH_2PO_4 per liter. 1.098 grams are made up to 100 cc., and 10 cc. of this stock solution are diluted to 1 liter.

Procedure

The calcium in 10 cc. of 1:5 trichloracetic acid serum filtrate is precipitated as described for the method of Halversen and Bergeim in the preceding chapter, and washed by the Clark and Collip technique there

outlined. The mother liquors and washings are decanted into a Pyrex test tube 16 by 150 mm. marked at 15 cc. 1 cc. of 2 per cent potassium dihydrogen phosphate and 1 cc. of concentrated ammonia solution are added. The precipitate is rubbed down from the sides of the tube with a rubber-tipped rod, the rod is rinsed into the tube, and the latter is let stand four hours to insure complete precipitation. Centrifuge ten minutes at 1500 r.p.m. and pour off the fluid. Add 20 cc. of the alcoholic wash solution, scrub the sides of the tube and stir up the precipitate with the rubber tipped rod. Centrifuge and decant the supernatant liquid again.

To the precipitate in the tube add 5 cc. of water and in a similar tube place 3 cc. of a standard potassium dihydrogen phosphate solution, containing 0.075 mg. of phosphorus. The phosphorus is then determined by one of the colorimetric methods (see p. 775–780).

Calculation

$$\frac{5.88\ S}{U\ V} \quad \text{milligrams of Mg in 100 cc. of serum.}$$

$$\frac{4.83\ S}{U\ V} \quad \text{milli-equivalents of Mg per liter of serum.}$$

S = reading of standard, U = reading of unknown, V = the volume in cubic centimeters of serum represented by the amount of filtrate taken for analysis.

BIBLIOGRAPHY

1. Briggs, A. P.: A colorimetric method for the determination of small amounts of magnesium. *J. Biol. Chem.*, 1922, **52**, 349.
2. Briggs, A. P.: Some applications of the colorimetric phosphate method. *J. Biol. Chem.*, 1924, **59**, 255.
3. Denis, W.: Determination of magnesium in blood. *J. Biol. Chem.*, 1920, **41**, 363.
4. Denis, W.: The determination of magnesium in blood, plasma, and serum. *J. Biol. Chem.*, 1922, **52**, 411.
5. Dienes, L.: Further studies on the determination of calcium, magnesium, and phosphorus in animal substances. *J. Biol. Chem.*, 1924, **61**, 77.
6. Fiske, C. H.: The determination of inorganic phosphorus in urine by alkalimetric titration. *J. Biol. Chem.*, 1921, **46**, 285.
7. Hammett, F. S., and Adams, E. T.: A colorimetric method for the determination of small amounts of magnesium. *J. Biol. Chem.*, 1922, **52**, 211.
   Note on a colorimetric method for the determination of small amounts of magnesium. *J. Biol. Chem.*, 1922, **54**, 565.
8. Hirth, A.: Le dosage du magnésium dans le plasma sanguin. *Compt. rend. soc. biol.*, 1923, **88**, 460.

9. JONES, W.: The admissibility of ammonium magnesium phosphate as a form in which to weigh phosphoric acid. *J. Biol. Chem.*, 1916, **25**, 87.

10. KOLTHOFF, J. M.: Eine neue spezifische Farbreaktion auf Magnesium und eine einfache kolorimetrische Methode zur quantitativen Bestimmung von Spuren dieses Elements. *Biochem. Z.*, 1927, **185**, 344.

11. KRAMER, B., AND TISDALL, F. F.: A simple technique for the determination of calcium and magnesium in small amounts of serum. *J. Biol. Chem.*, 1921, **47**, 475.

12. KRAMER, B., AND TISDALL, F. F.: The direct quantitative determination of sodium, potassium, calcium and magnesium in small amounts of blood. *J. Biol. Chem.*, 1921, **48**, 223.

13. MARRIOTT, W. MCK., AND HOWLAND, J.: A micro method for the determination of calcium and magnesium in blood serum. *J. Biol. Chem.*, 1917, **32**, 233.

14. MCCRUDDEN, F. H.: The quantitative separation of calcium and magnesium in the presence of phosphates and small amounts of iron devised especially for the analysis of foods, urine and feces. *J. Biol. Chem.*, 1909–10, **7**, 83.

15. MCCRUDDEN, F. H.: The determination of calcium in the presence of magnesium and phosphates: The determination of calcium in urine. *J. Biol. Chem.*, 1911–12, **10**, 187.

16. STOLBA, F.: Zur quantitativen Bestimmung der Magnesia, der Phosphorsäure, und Arsensäure durch Alkalimetrie. *Z. Anal. Chem.*, 1877, **16**, 100.

17. TISDALL, F. F., AND KRAMER, B.: Methods for the direct quantitative determination of sodium, potassium, calcium and magnesium in urine and stools. *J. Biol. Chem.*, 1921, **48**, 1.

CHAPTER XXVII

THE pH OF BLOOD AND URINE[1]

DISCUSSION

In 1917, Parsons (33) showed that the pH of whole blood measured with the hydrogen electrode is really the pH of the plasma. Methods for the determination of the pH of blood plasma or serum have been based for the most part on three principles:[2]

1. Electrometric determination with the hydrogen electrode. This is the classical method to which all others are referred for comparison. It was first applied by Höber (22). The first successful determinations of the pH of blood were made by Hasselbalch and Lundsgaard (16) and by Michaelis and Davidoff (28) in 1912. Since then, the technique has been further improved by W. Mansfield Clark (5), Cullen (6), and Erik Warburg (42).

Recently, two other electrometric methods have become available. Cullen and his co-workers (7, 8, 9, 12) have applied the quinhydrone electrode to serum and have determined the correction factor of this electrode at 20°. Kerridge (23) in 1925 demonstrated the application of the glass electrode to pH determinations of blood. MacInnes and Dole (27) have recently developed a type of glass electrode for use with small amounts of

[1] The authors are indebted to Dr. J. Sendroy, Jr., for preparing much of this chapter.

[2] For a discussion of the meaning of hydrogen ion concentration, and pH, and their significance in physiology and pathology, the reader is referred to the chapter on "Carbonic acid and acid-base balance" in volume I. For more complete descriptions of the principles and technique of pH determinations than can be given here, see the standard work on the subject by W. Mansfield Clark (5).

Evans (On a probable error in determinations by means of the hydrogen electrode, *J. Physiol.*, 1921–22, **54**, 353) reported difficulty in getting agreement between pH values determined in bicarbonate containing solutions by the hydrogen electrode and by the colorimetric method, and was inclined to conclude that in the presence of H_2CO_3 and $BHCO_3$ the electrometric measurements were in error by as much as 0.2 pH. Cullen and Hastings, however, (A comparison of colorimetric and electrometric determinations of hydrogen ion concentrations in solutions containing carbon dioxide, *J. Biol. Chem.*, 1922, **52**, 517) found that when precautions were taken to control the CO_2 tension, and phenol red was used as indicator, electrometric and colorimetric results agreed. They found no evidence of any disturbing effect of bicarbonate or carbonic acid on the accuracy of electrometric pH determinations. Their experience has apparently been confirmed by that of subsequent authors, since, so far as the writers are aware, none have reported difficulties in electrometric pH determinations in bicarbonate containing solutions.

material, and have demonstrated its specficity for hydrogen ions. Stadie, O'Brien, and Laug (38) have applied the MacInnes and Dole electrode especially to serum pH determinations.

2. Colorimetric determinations. While the hydrogen electrode provides the standard for all pH values, its demands in technique are such that it is hardly to be considered as a generally applicable clinical procedure. The colorimetric methods are simpler, and the most recent ones yield results almost as accurate as the electrometric. The blood or urine is handled to prevent CO_2 loss, and is diluted to eliminate as much as possible the error due to the color of the material itself and the effect of protein, when this is present. An indicator is added to the diluted sample, or a dialyzate of the original material, and the resulting color is matched against standards.

The colorimetric procedure depends on the behavior of certain substances, known as indicators, which change color when the pH of the solution is altered. The indicators referred to in this chapter show two colors, one in alkaline and another in acid solutions. At a certain pH which is characteristic for each indicator, half of the substance will be present in the alkaline form, half in the acid form. As the pH varies from this point, one or the other color predominates in accordance with Henderson's modification of the mass law equation,

$$\mathrm{pH} = \mathrm{pK}' + \log \frac{\text{alkaline form}}{\text{acid form}}$$

where pK′ is an approximate constant. The value of pK′, and hence the indicator color at a given pH, is affected by salts, proteins, etc. (See Clark (5) and Sendroy and Hastings (34).)

Standards with colors corresponding to those of solutions of definite pH over a given range may be prepared in two ways: 1. The indicator may be added in constant amount to a series of *standard buffer solutions of* known pH, so that each standard contains a mixture of the acid and alkaline forms of the indicator, the proportions of which may be calculated by the above equation from the pK′ of the indicator and the pH of the buffer solution. The original methods of Henderson and Palmer (21) for urine pH and of Cullen (6) for plasma pH were based on comparison with such standards.

2. Standards may be made by preparing a series of pairs of tubes, one containing alkali, the other acid. The indicator is distributed in varying proportions between the alkali and acid tube of each pair, so that when transmitted light is received through the two tubes, the resulting colors are those of mixtures of the alkaline and acid colors of the indicator. This "*bicolor*" method, introduced by Bjerrum (4) and utilized by Gillespie (14)

and Myers (29), has been applied by Hastings and Sendroy in modifying the Cullen method for estimating plasma pH. The bicolor standards have several advantages over the previously more commonly used buffer standards. The bicolor standards are not affected by temperature changes; they are much more permanent than buffer standards, which may change in a few days or weeks, even when kept cold and dark; the accuracy of the bicolor standards does not depend on the use of buffer salts of great purity. On the other hand, the accuracy of the bicolor standards does depend upon the use of dyes free from impurities which affect the color.

Therefore, the precaution of *testing the purity of the indicator should never be omitted when using bicolor standards*. A standard buffer solution is prepared with pH near that at which the indicator to be tested presents a good readable color. When possible, a pH near the pK′ of the indicator is preferable. Then into two tubes, one acid and one alkaline, are measured solutions of the indicator in such proportions, that they should, according to tables 61 to 65, transmit the same color as the buffer solution when an equal total concentration of the indicator is added to it. If the colors match, the indicator is satisfactorily pure. If there is a difference, the indicator should be rejected or the bicolor readings corrected, depending on the magnitude of the discrepancy.

The application of the colorimetric technique to urine was made by Henderson and Palmer (21) in 1912. They used phenolphthalein, neutral red, *p*-nitrophenol, and methyl red as indicators, with phosphate and acetate buffer standards. Hastings, Sendroy, and Robson (18) have applied Clark's indicators (5) to the bicolor technique, and have brought the method to an accuracy approaching the electrometric.

The application of the colorimetric technique to blood was made by Levy, Rowntree, and Marriott (25). They devised the ingenious procedure of dialyzing the blood against physiological salt solution and measuring colorimetrically the pH of the dialyzate. The technique was later improved by Dale and Evans (10), who added precautions to prevent loss of carbon dioxide and consequent increase of pH during the procedure. Lindhard (26) later modified the method for use with small amounts of blood.

Cullen (6) introduced the simpler direct colorimetric determination on serum or plasma diluted 20-fold with neutral saline solution. The pH determined at room temperature, by comparison of dilute plasma + phenol red with standard solutions of buffer + phenol red, was found by Cullen to be higher than the pH determined in the plasma electrometrically at body temperature. The difference was partly due to temperature and partly to the effect of proteins and perhaps other substances in the plasma

on the phenol red. The total difference between the colorimetric pH determined at 20° and the electrometric pH at 38° was determined by Cullen for plasma of several species. This difference is subtracted as a correction to the observed colorimetric pH in order to find the true pH of the plasma in the circulating blood. The correction was found to be 0.22, 0.17, and 0.12 pH for human, rabbit, and horse plasma, respectively. For horse serum the correction was 0.16, distinctly higher than the correction for plasma from the same horse. The correction for dog serum averages 0.35. The correction for human plasma decreases by 0.01 pH for every 1° increase of temperature between 20° and 30°. Although the empirical correction factor seems quite constant for plasma from normal men it has been found to vary considerably in some pathological conditions (3). Austin, Stadie, and Robinson (3) therefore proposed that the empirical correction factor for each subject whose plasma was studied should be determined by simultaneous colorimetric and gasometric determination on a sample of plasma brought to a known CO_2 tension.

Hastings and Sendroy (17) added several improvements to Cullen's method. They adapted to it the bicolor standards, and, by making the color comparisons at 38° instead of room temperature they eliminated, save in exceptional pathological cases, the necessity for the use of empirical corrections with human plasma.

3. Gasometric determinations. By use of the Henderson-Hasselbalch equation (15)

$$\mathrm{pH} = \mathrm{pK}' + \log \frac{\mathrm{BHCO_3}}{\mathrm{H_2CO_3}} = \mathrm{pK}' + \log \frac{\mathrm{CO_2} - 0.1316\,\alpha p}{0.1316\,\alpha p}$$

pH can be calculated from the total volumes per cent carbon dioxide, CO_2, the CO_2 tension, p, and the CO_2 solubility, α (p. 308). (For discussion of the Henderson-Hasselbalch equation see pages 874–882 of volume I.) Various authors have applied the procedure to estimate the pH_s of circulating blood. They have constructed CO_2 absorption curves of the blood and interpolated on them the CO_2 content found in a sample of the freshly drawn blood. (For discussion of CO_2 absorption curves of blood see chapter on "Carbonic acid and acid-base balance" in volume I). Because of the formation of lactic acid in whole blood during the process of saturation with air of known CO_2 content at 38° to obtain the absorption curves, because of the continual CO_2 production that goes on in whole blood, and because of the difficulty of precise correction for the effect of degree of oxygen saturation on the CO_2 and pH of whole blood, extraordinary precautions are necessary in order to obtain accurate results by the gasometric pH method ap-

plied to whole blood. Although some authors have been successful (see p. 913, volume I), it appears certain that the majority of pH estimations made by interpolation on the CO_2 absorption curves of whole blood have not yielded tenable results.

When, however, plasma or serum is separated anaerobically from the freshly drawn blood and used for the interpolation method the above sources, of error, encountered with whole blood, are avoided. Eisenman (13) has accordingly been able to apply the gasometric interpolation method successfully to plasma and serum. Her method is described in the gasometric chapter on page 298.

Van Slyke, Sendroy, and Liu have determined the CO_2 tension of blood by equilibrating for a short time a relatively large volume with a small bubble of air already in approximate equilibrium with the CO_2 and O_2 tension of the blood. The final CO_2 tension of the air in the bubble is determined by micro gas analysis in the Van Slyke-Neill manometric apparatus, and is used to calculate the pH by the Henderson-Hasselbalch equation. This method is described on page 309 in the gasometric chapter.

URINE pH. BICOLOR METHOD OF HASTINGS, SENDROY, AND ROBSON (18)

Reagents

0.01 N sodium hydroxide and *hydrochloric acid.*

0.001 N hydrochloric acid and 0.001 N sodium hydroxide are made by dilution from the above.

0.1 per cent cresol red solution.

0.1 per cent phenol red solution.

0.1 per cent brom cresol purple solution.

0.1 per cent brom cresol green solution.

The 0.1 per cent stock indicator solutions are made from the solid dyes by dissolving with the aid of the amounts of alkali indicated in table 60 and diluting to 100 cc.

As pointed out by Hastings, Sendroy, and Robson (18), chlor phenol red is of but limited use, and we have omitted it in the tables. Cresol red, not used in the original paper (18) has been added because of its usefulness in the higher alkalinities. The pK′ of this indicator has been investigated by Davis and Hastings (11), who have been kind enough to supply, previous to publication, the data on which table 61 for the preparation of cresol red standards is based.

Procedure

The urine is collected and kept under oil. As soon as possible after collection, 2 cc. are pipetted into a test tube containing 1 cc. of indicator

TABLE 60

INDICATOR SOLUTIONS FOR URINE pH

INDICATOR	TO MAKE 100 CC. OF STOCK SOLUTION			VOLUME OF STOCK SOLUTION DILUTED TO 200 CC. TO MAKE INDICATOR SOLUTION USED	FINAL CONCENTRATION OF INDICATOR
	Indicator	Sodium hydroxide			
		0.05 N solution	Equivalents per mole indicator		
	gram	*cc.*		*cc.*	*per cent*
Cresol red	0.1	5.3	1.0	16	0.008
Phenol red	0.1	5.7	1.0	15	0.0075
Brom cresol purple	0.1	4.1	1.1	16	0.008
Brom cresol green	0.1	3.2	1.1	32	0.016

TABLE 61

BICOLOR STANDARDS OF CRESOL RED FOR URINE pH 7.6 TO 8.6

Table for preparation of bicolor standards, with 0.008 per cent cresol red, 0.001 N HCl, and 0.01 N NaOH. Cresol red pK′ = 8.22 at 21°. (Based on data of Davis and Hastings (11).)

$pH_{21°}$	ALKALI TUBE		ACID TUBE	
	Dye	Alkali	Dye	Acid
	cc.	*cc.*	*cc.*	*cc.*
7.60	0.49	24.60	2.01	22.99
7.70	0.58	24.51	1.92	23.08
7.80	0.69	24.42	1.81	23.19
7.90	0.81	24.31	1.69	23.31
8.00	0.94	24.19	1.56	23.44
8.10	1.08	24.06	1.42	23.58
8.20	1.23	23.92	1.27	23.73
8.30	1.38	23.77	1.12	23.88
8.40	1.51	23.62	0.99	24.01
8.50	1.64	23.49	0.86	24.14
8.60	1.77	23.36	0.73	24.27

solution and 7 cc. of redistilled water under oil. During the delivery the pipette tip should dip beneath the solution in the tube, so that the urine will not stream through air and lose CO_2. For a control, another

plied to whole blood. Although some authors have been successful (see p. 913, volume I), it appears certain that the majority of pH estimations made by interpolation on the CO_2 absorption curves of whole blood have not yielded tenable results.

When, however, plasma or serum is separated anaerobically from the freshly drawn blood and used for the interpolation method the above sources, of error, encountered with whole blood, are avoided. Eisenman (13) has accordingly been able to apply the gasometric interpolation method successfully to plasma and serum. Her method is described in the gasometric chapter on page 298.

Van Slyke, Sendroy, and Liu have determined the CO_2 tension of blood by equilibrating for a short time a relatively large volume with a small bubble of air already in approximate equilibrium with the CO_2 and O_2 tension of the blood. The final CO_2 tension of the air in the bubble is determined by micro gas analysis in the Van Slyke-Neill manometric apparatus, and is used to calculate the pH by the Henderson-Hasselbalch equation. This method is described on page 309 in the gasometric chapter.

URINE pH. BICOLOR METHOD OF HASTINGS, SENDROY, AND ROBSON (18)

Reagents

0.01 N sodium hydroxide and *hydrochloric acid.*

0.001 N hydrochloric acid and 0.001 N sodium hydroxide are made by dilution from the above.

0.1 per cent cresol red solution.

0.1 per cent phenol red solution.

0.1 per cent brom cresol purple solution.

0.1 per cent brom cresol green solution.

The 0.1 per cent stock indicator solutions are made from the solid dyes by dissolving with the aid of the amounts of alkali indicated in table 60 and diluting to 100 cc.

As pointed out by Hastings, Sendroy, and Robson (18), chlor phenol red is of but limited use, and we have omitted it in the tables. Cresol red, not used in the original paper (18) has been added because of its usefulness in the higher alkalinities. The pK′ of this indicator has been investigated by Davis and Hastings (11), who have been kind enough to supply, previous to publication, the data on which table 61 for the preparation of cresol red standards is based.

Procedure

The urine is collected and kept under oil. As soon as possible after collection, 2 cc. are pipetted into a test tube containing 1 cc. of indicator

TABLE 60

INDICATOR SOLUTIONS FOR URINE pH

INDICATOR	TO MAKE 100 CC. OF STOCK SOLUTION			VOLUME OF STOCK SOLUTION DILUTED TO 200 CC. TO MAKE INDICATOR SOLUTION USED	FINAL CONCENTRATION OF INDICATOR
	Indicator	Sodium hydroxide			
		0.05 N solution	Equivalents per mole indicator		
	gram	*cc.*		*cc.*	*per cent*
Cresol red	0.1	5.3	1.0	16	0.008
Phenol red	0.1	5.7	1.0	15	0.0075
Brom cresol purple	0.1	4.1	1.1	16	0.008
Brom cresol green	0.1	3.2	1.1	32	0.016

TABLE 61

BICOLOR STANDARDS OF CRESOL RED FOR URINE pH 7.6 TO 8.6

Table for preparation of bicolor standards, with 0.008 per cent cresol red, 0.001 N HCl, and 0.01 N NaOH. Cresol red pK′ = 8.22 at 21°. (Based on data of Davis and Hastings (11).)

$pH_{21°}$	ALKALI TUBE		ACID TUBE	
	Dye	Alkali	Dye	Acid
	cc.	*cc.*	*cc.*	*cc.*
7.60	0.49	24.60	2.01	22.99
7.70	0.58	24.51	1.92	23.08
7.80	0.69	24.42	1.81	23.19
7.90	0.81	24.31	1.69	23.31
8.00	0.94	24.19	1.56	23.44
8.10	1.08	24.06	1.42	23.58
8.20	1.23	23.92	1.27	23.73
8.30	1.38	23.77	1.12	23.88
8.40	1.51	23.62	0.99	24.01
8.50	1.64	23.49	0.86	24.14
8.60	1.77	23.36	0.73	24.27

solution and 7 cc. of redistilled water under oil. During the delivery the pipette tip should dip beneath the solution in the tube, so that the urine will not stream through air and lose CO_2. For a control, another

TABLE 62

BICOLOR STANDARDS OF PHENOL RED FOR URINE pH 6.7 TO 8.2

Table for preparation of bicolor standards with 0.0075 per cent phenol red, 0.001 N HCl, and 0.01 N NaOH. Phenol red pK′ = 7.65 at 38°, and 7.78 at 20°. (Hastings, Sendroy and Robson (18).)

$pH_{38°}$	ALKALI TUBE		ACID TUBE		$pH_{20°}$
	Dye	Alkali	Dye	Acid	
	cc.	*cc.*	*cc.*	*cc.*	
6.70	0.25	24.75	2.25	22.75	6.83
6.80	0.31	24.69	2.19	22.81	6.93
6.90	0.38	24.62	2.12	22.88	7.03
7.00	0.46	24.54	2.04	22.96	7.13
7.10	0.55	24.45	1.95	23.05	7.23
7.20	0.65	24.35	1.85	23.15	7.33
7.30	0.77	24.23	1.73	23.27	7.43
7.40	0.90	24.10	1.60	23.40	7.53
7.50	1.04	23.96	1.46	23.54	7.63
7.60	1.18	23.82	1.32	23.68	7.73
7.70	1.32	23.68	1.18	23.82	7.83
7.80	1.46	23.54	1.04	23.96	7.93
7.90	1.60	23.40	0.90	24.10	8.03
8.00	1.73	23.27	0.77	24.23	8.13
8.10	1.85	23.15	0.65	24.35	8.23
8.20	1.95	23.05	0.55	24.45	8.33

TABLE 63

BICOLOR STANDARDS OF BROM CRESOL PURPLE FOR URINE pH 5.6 TO 6.9

Table for preparation of bicolor standards with 0.008 per cent brom cresol purple, 0.002 N HCl, and 0.01 N NaOH. Brom cresol purple pK′ = 6.09 at 38°, and 6.19 at 20°. (Hastings, Sendroy and Robson (18).)

$pH_{38°}$	ALKALI TUBE		ACID TUBE		$pH_{20°}$
	Dye	Alkali	Dye	Acid	
	cc.	*cc.*	*cc.*	*cc.*	
5.60	0.61	24.39	1.89	23.11	5.70
5.70	0.72	24.28	1.78	23.22	5.80
5.80	0.85	24.15	1.65	23.35	5.90
5.90	0.99	24.01	1.51	23.49	6.00
6.00	1.12	23.88	1.38	23.62	6.10
6.10	1.26	23.74	1.24	23.76	6.20
6.20	1.40	23.60	1.10	23.90	6.30
6.30	1.55	23.45	0.95	24.05	6.40
6.40	1.68	23.32	0.82	24.18	6.50
6.50	1.80	23.20	0.70	24.30	6.60
6.60	1.91	23.09	0.59	24.41	6.70
6.70	2.01	22.99	0.49	24.51	6.80
6.80	2.09	22.91	0.41	24.59	6.90
6.90	2.16	22.84	0.34	24.66	7.00

portion of urine is run into a similar test tube (22 by 175 mm.) with 8 cc. of water. After gentle stirring with a footed rod, the unknown solution is brought to 38° in a water bath and matched with pairs of standard indicator solutions prepared according to tables 61, 62, 63, and 64. To

TABLE 64

BICOLOR STANDARDS OF BROM CRESOL GREEN FOR URINE pH 4.0 TO 5.8

Table for preparation of bicolor standards with 0.016 per cent brom cresol green, 0.002 N HCl, and 0.001 N NaOH. Brom cresol green pK′ = 4.72 at 38° and 20°. (Hastings, Sendroy and Robson (18).)

$pH_{38° \text{ and } 20°}$	ALKALI TUBE		ACID TUBE	
	Dye	Alkali	Dye	Acid
	cc.	cc.	cc.	cc.
4.00	0.40	24.60	2.10	22.90
4.10	0.49	24.51	2.01	22.99
4.20	0.58	24.42	1.92	23.08
4.30	0.69	24.31	1.81	23.19
4.40	0.81	24.19	1.69	23.31
4.50	0.94	24.06	1.56	23.44
4.60	1.08	23.92	1.42	23.58
4.70	1.23	23.77	1.27	23.73
4.80	1.38	23.62	1.12	23.88
4.90	1.51	23.49	0.99	24.01
5.00	1.64	23.36	0.86	24.14
5.10	1.77	23.23	0.73	24.27
5.20	1.88	23.12	0.62	24.38
5.30	1.98	23.02	0.52	24.48
5.40	2.07	22.93	0.43	24.57
5.50	2.14	22.86	0.36	24.64
5.60	2.21	22.79	0.29	24.71
5.70	2.26	22.74	0.24	24.76
5.80	2.31	22.69	0.19	24.81

allow for the error introduced by the dilution of the urine, 0.10 pH is subtracted from the reading.[3] This corrected pH has been found to be

[3] If the color readings are made with the diluted urine at room temperature instead of 38° the correction is greater. Myers and Muntwyler (30) found that when the readings were made with the diluted urine at 25° the following mean corrections were obtained, varying somewhat with the indicator used: Phenol red, 0.24; brom thymol blue, 0.20; brom cresol purple, 0.20; brom cresol green, 0.22. These amounts are subtracted from the colorimetric pH read at 25° to give the electrometric pH at 38°.

Myers and Muntwyler (30) found that their colorimetric results were somewhat more

within 0.05 of the electrometric pH at the same temperature. The indicators should be tested as described above on page 790. The readings are made in a comparator (figure 87) as described on page 799.

PLASMA pH. HASTINGS AND SENDROY'S (18) BICOLOR MODIFICATION OF CULLEN'S (6) METHOD

Reagents

0.01 N sodium hydroxide.

0.001 N hydrochloric acid.

0.1 per cent phenol red stock solution prepared as described in table 60.

0.0075 per cent phenol red for standard solutions, made by diluting 15 cc. of the 0.1 per cent solution to 200 cc. with redistilled water.

Adjusted saline phenol red solution for dilution of blood or plasma. This solution, which contains per liter 0.154 M NaCl and 0.0000222 M phenol red, is prepared as follows: 0.9 gram of NaCl is dissolved in freshly redistilled water in a 100-cc. flask. 10.5 cc. of 0.0075 per cent phenol red solution are added if the saline is to be used in plasma determinations, 11.0 cc. if it is to be used for whole blood. The mixture is then diluted to the 100-cc. mark. A 0.154 M solution of NaCl without phenol red is prepared at the same time for control tubes.

The saline solution is adjusted to the approximate blood reaction as follows: At the beginning of each experiment, after addition of the phenol red, as indicated above, the solution is covered with paraffin oil. 0.01 N NaOH is admitted by capillary pipette, with stirring, until the solution attains a pH of 7.4 ±0.2. Saline solution should be adjusted in this manner the same day that it is used.

Bicolor standards. Each pH standard is made by measuring from a microburette into each of a pair of test tubes (22 by 175 mm.) an amount of 0.0075 per cent phenol red solution indicated by the equation

$$pH_{38^\circ} = 7.65 + \log \frac{\text{alkaline form}}{\text{acid form}},$$

consistently related to the electrometric when they diluted the urine with saline solution of known pH instead of distilled water. The saline solution was mixed with indicator, and the pH was brought to a definite point by addition of minute amounts of NaOH or HCl before the solutions were used. The pH of the indicator + saline solution varied with the indicator as follows: Phenol red, pH 7.4; brom thymol blue, pH 7.2; brom cresol purple, pH 6.2; brom cresol green, pH 5.0. These solutions are kept in glass stoppered Pyrex bottles. The degree of agreement with electrometric results at 38° when the urine is diluted with these saline solutions and the colorimetric reading at room temperature is corrected as in the preceding paragraph, appears to be about the same as in the simpler Hastings-Sendroy-Robson method, the error being usually within ± 0.05 pH.

then diluting to 25 cc. with 0.001 N HCl or 0.01 N NaOH. The amounts of indicator solution required are given in table 65. For blood plasma

TABLE 65

BICOLOR STANDARDS OF PHENOL RED FOR PLASMA pH 6.7 TO 8.0

pH values at 38° at 0.05 intervals, with corresponding amounts of 0.0075 per cent phenol red and 0.01 N NaOH or 0.001 N HCl.* (From Hastings and Sendroy (17).) Phenol red pK′ = 7.65 at 38°.

pH	ALKALI TUBE		ACID TUBE	
	Dye	Alkali	Dye	Acid
	cc.	cc.	cc.	cc.
6.70	0.25	24.75	2.25	22.75
6.75	0.28	24.72	2.22	22.78
6.80	0.31	24.69	2.19	22.81
6.85	0.34	24.66	2.16	22.84
6.90	0.38	24.62	2.12	22.88
6.95	0.42	24.58	2.08	22.92
7.00	0.46	24.54	2.04	22.96
7.05	0.50	24.50	2.00	23.00
7.10	0.55	24.45	1.95	23.05
7.15	0.60	24.40	1.90	23.10
7.20	0.65	24.35	1.85	23.15
7.25	0.71	24.29	1.79	23.21
7.30	0.77	24.23	1.73	23.27
7.35	0.84	24.16	1.66	23.34
7.40	0.90	24.10	1.60	23.40
7.45	0.97	24.03	1.53	23.47
7.50	1.04	23.96	1.46	23.54
7.55	1.11	23.89	1.39	23.61
7.60	1.18	23.82	1.32	23.68
7.65	1.25	23.75	1.25	23.75
7.70	1.32	23.68	1.18	23.82
7.75	1.39	23.61	1.11	23.89
7.80	1.46	23.54	1.04	23.96
7.85	1.53	23.47	0.97	24.03
7.90	1.69	23.40	0.90	24.10
7.95	1.67	23.33	0.82	24.17
8.00	1.73	23.27	0.77	24.23

* Originally 0.0001 N HCl was used (17) but Hastings and Sendroy (personal communication) have since found 0.001 N more practicable.

determinations only the standards covering the pH range from 7.10 to 7.60 are usually required, but extreme cases may reach 7.00 or 7.80.

After the standards are prepared, 4-cc. portions are transferred to the smaller tubes (15 by 100 mm.) used for the serum pH determinations. These tubes should have the open ends previously drawn out. After transfer of the solutions, the tubes are sealed off. They are kept in the dark when not in use. Under these conditions standards have been found to remain stable for several months. *From time to time they should be checked against standard phosphate solutions* prepared according to table 70.

Drawing blood samples

Two procedures may be used: one in which the plasma or serum is separated before it is diluted with saline indicator solution, and one in which the whole blood, oxalated or defibrinated, is directly diluted with saline and then centrifuged. This latter procedure, introduced by Hawkins (20), may be carried out with the least amount of blood. In either case, it is the ***plasma*** or ***serum*** pH that is estimated.

With either procedure it is essential to use blood which has not had its carbon dioxide content augmented by stasis nor diminished by exposure to air, and which has not been permitted to form lactic acid *in vitro* by glycolysis. All the precautions (2) described in the section on the preparation of blood for gas studies must be observed (see chapter II).

To prevent possibility of the sudden pH_s fall of about 0.03 pH reported by Havard and Kerridge (19), and Laug (24), the blood would have to be instantly cooled to 0°. This precaution has not, however, been taken in obtaining any of the clinical data in the literature, and is unnecessary when the object is to obtain pH_s values to compare with those data.[4]

[4] Havard and Kerridge (19) with the glass electrode and Laug (24) with the quinhydrone electrode have observed an apparent sudden fall, averaging 0.03 pH, which occurs in 6 minutes in shed blood kept at 38°. It apparently occurs before glycolysis has formed enough lactic acid to cause such a pH change. The pH fall requires 1.5 to 2 hours to occur in blood immediately cooled to 20°, and is inappreciable at 0°. The apparent pH drop at 38° is not prevented by sodium fluoride, KCN, thymol, or anticoagulants, and the chemical cause is yet unexplained. Gasometric pH determinations, by Hasselbalch's method, do not show evidence of such a pH change, for they have been found to yield CO_2 tensions agreeing with those of the alveolar air (see "Gasometric pH," chapter VII). The apparent sudden pH drop in shed blood is therefore at present something of a mystery. However, even if it is a genuine pH change, it does not seriously affect the clinical significance of pH determinations on plasma of blood drawn and centrifuged at room temperature, since they are to be compared with pH values in the literature arrived at by similar technique. Consequently, even though all such pH values should be about 0.03 lower than those in the circulating blood, the relative values in health and disease would not be greatly affected if subsequent correction by this amount proved necessary.

Dilution of plasma and pH determination at 38°

Into a small test tube (15 by 100 mm.), 4 cc. of adjusted saline phenol red solution are pipetted under oil. 0.2 cc. of plasma is added and the mixture is gently stirred with a footed glass rod. A control tube is prepared in a similar manner, with plasma and saline solution, but without indicator. The oil in the colored tube is replaced by a layer of low-melting paraffin (45°), to prevent escape of CO_2 while the tube is heated to body temperature. For the latter procedure, both the tube of plasma + dye and the control are immersed in a beaker of water with a temperature of about 45°. A thermometer is placed in the control tube. When it indicates 39°, both tubes are withdrawn from the bath and the colorimeter reading is carried out as quickly as possible.

The tubes are placed in a comparator block arranged as in figure 87. One should previously place in the row to be occupied by the control tube the pair of standard tubes containing acid and alkaline dye in proportions

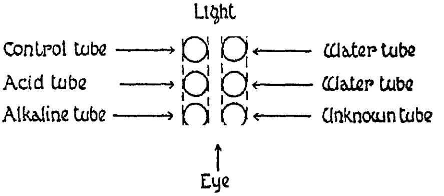


FIG. 87. Arrangement of tubes in comparator for reading colorimetric pH of blood by means of the bicolor standards (Hastings and Sendroy (17)).

to approximate the color which corresponds to the pH expected, 7.35 for example. The three tubes are arranged in a row so that the light observed passes through all of them. In a row with the colored tube of plasma + indicator are placed at the same time two tubes of water. If the color of the plasma-indicator solution is not matched, another pair of standard tubes, for pH 7.30 or 7.40, is substituted, and so on until the colors match. If the temperature in the control tube falls below 37° before the final reading is made, both the control and the tube with plasma + dye are again warmed to 39° and the readings repeated. The other 4 tubes are throughout used at room temperature.

pH_s determination on plasma at room temperature with correction. It is desirable for maximum accuracy to use the above technique, in which the plasma + saline + dye solution is brought to 38° for the colorimetric reading. In case it is inconvenient to do so, however, an

alternative procedure is to make the color comparison with the plasma solution at room temperature, and subtract a correction for the effect of temperature upon the apparent pH of the saline plasma solution.

In this case one employs the figures for pH values of the standard solutions that are given for $pH_{20}°$ in the right hand column of table 62, and *subtracts* from the result obtained the correction indicated by table 66.

Cullen (6) found that the pH of 20:1 saline solution of human plasma determined at 20° with phenol red by comparison with phosphate standards was 0.22 higher than the pH of the plasma determined by the gas chain method at 38°, and prescribed the subtraction of 0.22 pH from the 20° pH as a correction in order to estimate the pH at body temperature. He found that the correction decreased approximately 0.01 pH for each degree temperature rise between 20° and 30°. When, instead of phosphate standards, however, the bicolor standards of Hastings and Sendroy (17) for phenol red at 20° (Table 62, right hand column) are used there is, with increasing temperature above 20°, a partially compensatory correction in the opposite direction. This effect amounts to about 0.005 pH per 1° temperature increase. It is due to the fact that the pK′ of phenol red in the relatively salt-free solutions used in the bicolor method decreases with rising temperature, being 7.78 at 20° and 7.65 at 38°. Hence when the bicolor method is used at room temperature, with the pH values for 20° given in table 62, the correction changes only 0.005 pH for each degree of temperature change.

TABLE 66

TABLE FOR CORRECTION OF pH READ AT $t°$ TO pH AT 38°C. WHEN HASTINGS-SENDROY BICOLOR STANDARDS FOR 20° FROM TABLE 62 ARE USED

t°C.	CORRECTION	t°C.	CORRECTION
18	−0.230	27	−0.185
19	−0.225	28	−0.180
20	−0.220	29	−0.175
21	−0.215	30	−0.170
22	−0.210	31	−0.165
23	−0.205	32	−0.160
24	−0.200		
25	−0.195	38	−0.130
26	−0.190		

PLASMA pH, COLORIMETRIC, WITH A SAMPLE OF WHOLE BLOOD. HAWKINS (20)

Instead of 0.2 cc. of plasma or serum, 0.4 cc. of whole blood is taken as a sample. It is run under 4 cc. of the adjusted saline solution which contains 11 cc. of 0.0075 per cent phenol red solution per 100 cc. The

mixture is stirred under oil, the oil is replaced by a layer of paraffin and the tube is centrifuged. A control tube of blood-saline mixture is prepared in the same way, except that the indicator and paraffin are omitted. Both tubes are warmed to 39°, with precautions to avoid stirring up the layer of cells in the bottom of each. The pH readings should then be made, as described above, no later than one-half hour after centrifugation.

BICOLOR pH DETERMINATIONS WITH THE HASTINGS-DUBOSCQ COLORIMETER

(*Personal communication from A. Baird Hastings*)

In order to make possible pH determinations with the optical refinements of a colorimeter, Myers in 1922 (29) adapted the Hellige colorimeter to use with indicator solutions. The Myers bicolorimeter was successfully used in studying the pH of the blood (32, 31) and urine (30). Wedges containing the acid and alkaline forms of the indicator are used, and a third wedge is available which enables one, when necessary, to insert behind the indicator solutions a layer of the unknown solution in order to counterbalance any color or turbidity of its own. If the authors fail to describe the Myers apparatus, it is not for lack of appreciation of its utility, but because they must admit a prejudice in favor of the Duboscq type of colorimeter, which has been applied to the same use by Wu (43) and Hastings. The Duboscq is, we believe, an instrument with which exact color comparisons are somewhat more easily obtained, and in pH determinations it permits one to calculate results theoretically from the measured thicknesses of the alkaline and acid indicator layers, whereas the Hellige colorimeter requires the use of an empirical curve for each indicator.

In order to adapt the Duboscq to pH determinations Wu (43) in 1923 attached an additional movable cup to one side of the colorimeter, so that the observer could look through two solutions (the acid and alkaline indicator) and adjust at will the depths of each. As in the Myers apparatus, not only were the refinements of a colorimeter rendered available, but the necessity of making numerous standard solutions of the indicators was avoided. Only one acid and one alkaline solution of each indicator was required.

To make the Duboscq instrument available for bicolor pH determinations in liquids like blood plasma, where the color of the liquid itself necessitates placing a layer of it in series with the standard dye solutions, Hastings added to the Wu instrument the two auxiliary cups, H and H', shown in figure 88. In one of these cups is placed the control solution of plasma

without dye, while in the other is placed the plasma solution with dye. The cups *H* and *H'* are set into a metal water jacket (not shown in the

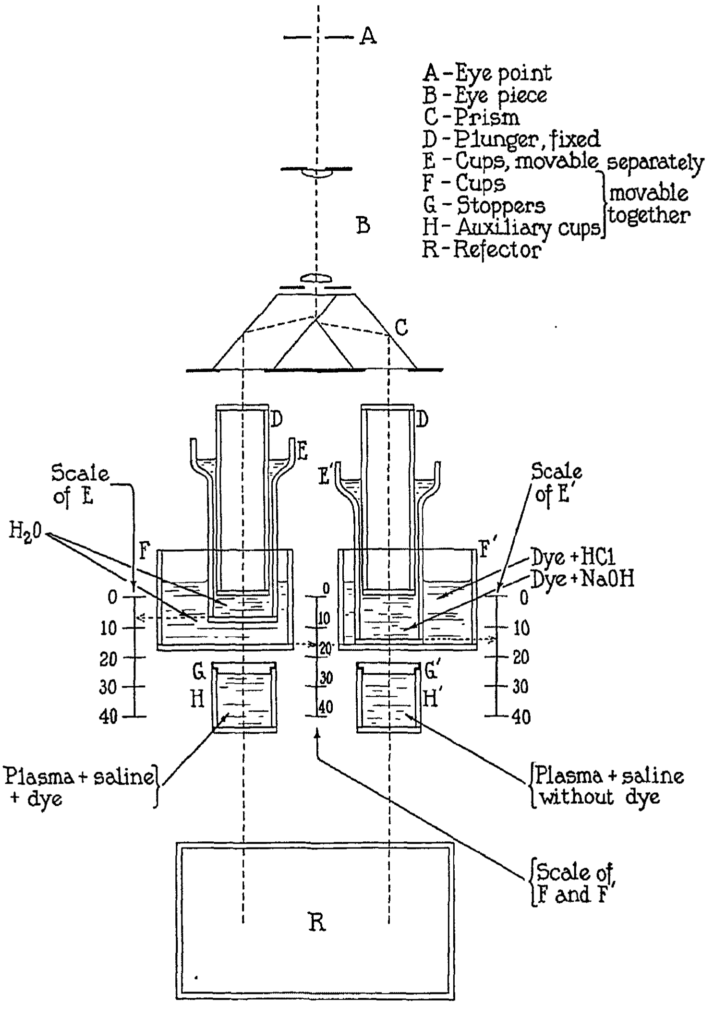


FIG. 88. Hastings colorimeter for the bicolorimetric determination of pH

diagram of figure 88) through which a circulation of water can be maintained to keep the temperature in *H* and *H'* at 38° if desired.[5] These cups are

[5] The Hastings apparatus is manufactured by the Bausch and Lomb Optical Company of Rochester, New York.

closed by glass covers, so that no CO_2 can escape from plasma or urine during a determination.

Reagents

The reagents required are those listed above for the determination of pH in urine and plasma. The same indicators detailed in table 60 are used for urine.

The standard tubes with measured amounts of dye solutions listed in tables 61, 62, 63, and 64 for urine and 65 for plasma are not needed with the colorimeter.

Procedure

The mixture of urine and water solution of indicator, or of plasma and saline solution of indicator, is made as described for the preceding methods, and is transferred without any oil to cup *H*, which is at once covered with the glass stopper. The control solution of urine or plasma without indicator is placed in cup *H′* and covered with the stopper.

For plasma pH cup *F′* is half filled with 0.001 N HCl and 0.0075 per cent phenol red in the proportions 9:1, and cup *E′* is half filled with 0.01 N NaOH and 0.0075 per cent phenol red in the same proportions. For urine pH the acid and alkaline dye solutions are similarly arranged. Cups *F* and *E* on the other side are half filled with water.

Cups *F* and *F′* are raised till their common pointer is on the 15-mm. mark of their scale. Then when *E′* is set also at its 15-mm. mark, as shown in figure 88, the light passes through 15 mm. of alkaline dye solution and none of the acidified dye solution. As *E′* is raised a layer of acid solution forms under it, while the layer of alkaline solution under the plunger in *E′* becomes more shallow.

Cup *E′* is moved up and down until a color match is obtained. The position of cup *E* is immaterial.

Calculation

$$\text{pH} = \text{pK}' + \log \frac{\text{reading of cup } E'}{15 - (\text{reading of cup } E')}$$

The values of pK′ for the different indicators are given in the headings of tables 61, 62, 63, 64, and 65.

If the readings are taken with the diluted plasma at room temperature calculations are made with pK′ of 7.78 for phenol red. From pH values thus calculated the corrections given above in table 66 are subtracted in order to estimate pH_s at 38°.

MICRO METHOD OF SHOCK AND HASTINGS FOR COMBINED DETERMINATION OF PLASMA pH AND TOTAL CO_2 IN 0.1 CC. OF BLOOD (35)[6]

Shohl (36) and Shock and Hastings (35) have independently devised micro methods in which small samples of plasma or blood are diluted in special pipettes in which the pH of the solution can be determined colorimetrically, and from which the solution can be transferred to the chamber of the Van Slyke-Neill manometric blood gas apparatus for determination of CO_2.

The method of Shock and Hastings will be described here, since it uses even less material than that of Shohl, and includes a determination of the cell volume in the blood. In the special pipette shown in figure 89 0.1 cc. of whole blood is measured, diluted 20-fold with neutral saline solution, and centrifuged. The pH is then read in the clear supernatant solution in the broad upper portion, as in Hawkins' method (p. 800). The blood together with the saline that has been used to dilute it is then transferred to the manometric blood gas apparatus and the CO_2 content is determined by the micro technique of Van Slyke and Neill. Blood with 50 volume per cent of CO_2 gives on analysis about 72 mm. of CO_2 pressure to read; the analysis is accurate to about 1 volume per cent or 0.4 mM. of CO_2, which suffices for nearly all studies of factors affecting the acid-base balance.

Apparatus

The apparatus is shown in figure 89. The *pipette* consists of a lower stem of about 1-mm. bore, a bulb of about 11-mm. inner diameter, and an upper stem of about 1-mm. bore. The lower stem is calibrated to contain (not to deliver, see calibration in chapter I) 0.1 cc., and is divided into 0.01-cc. divisions. It is essential that the bulb be made of the same kind of glass as the tubes containing the Hastings-Sendroy bicolor pH standards. The pipettes are made in Hastings' laboratory by sealing 0.2–cc. serological pipettes to test tubes similar in glass and diameter to those used for pH standards. The end of the test tube opposite the sealed on pipette is then drawn down to the capillary on which the 2-cc. mark is placed. The serological pipette is cut off at the 0.1-cc. mark and the end is beveled as shown in figure 89 by grinding. The pipette is accurately calibrated at 0.1 and 2 cc. by weighing the amount of mercury which the dry pipette holds. For closing the pipette during centrifugation large rubber bands are used.[7]

[6] The writers are indebted to Shock and Hastings for a somewhat more complete description of their method than that contained in the original publication (35).

[7] Pipettes of the design described may be obtained from the A. H. Thomas Company of Philadelphia. For the rubber bands Eberhard Faber No. 84 are satisfactory.

For collecting the blood obtained by skin puncture a *conical receiving vessel* (fig. 89) is made by sealing off the stem of a $1\frac{1}{2}$-inch funnel.

A *comparator block* (*C*, fig. 89) is used which is the same in principle as that shown in figure 87. In order to hold the Shock-Hastings pipettes, however, the holes across one row are drilled clear through the block, so that the graduated stems of the pipettes may extend through, permitting the bulbs to rest at the same levels as the tubes with standard indicator solutions. For convenience it is desirable to place the comparator block on legs.

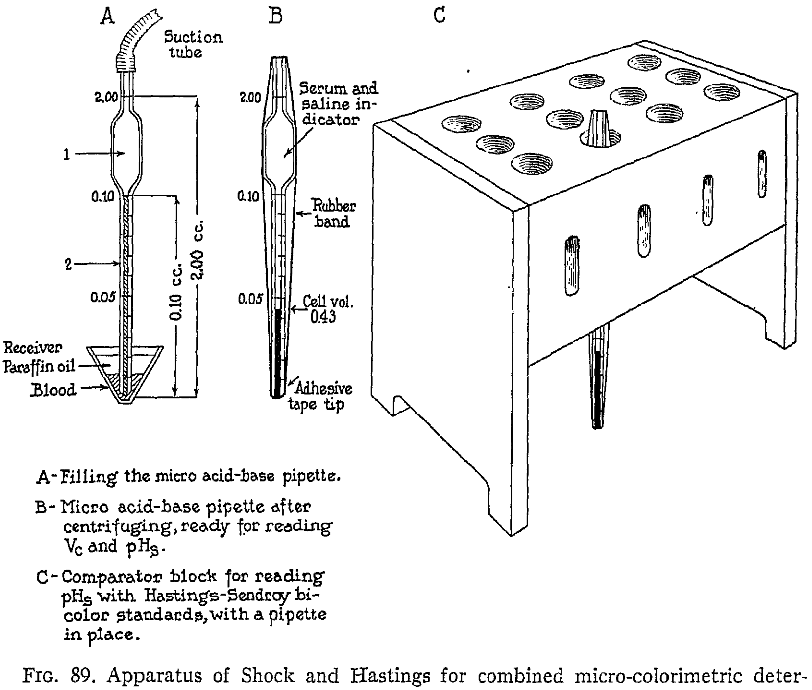


FIG. 89. Apparatus of Shock and Hastings for combined micro-colorimetric determination of pH and gasometric determination of CO_2 of blood.

Reagents

1. *Adjusted saline phenol red solution.* This is prepared as described on page 796 for the Hastings-Sendroy method, and the pH is adjusted to 7.4 immediately before use.

2. *Indicator-free saline solution (0.154* M). Nine grams of NaCl are dissolved without phenol red in a liter of water, for the control tubes.

3. *Neutral paraffin oil.*

4. *Paraffin oil saturated with a gas mixture of 5 per cent CO_2 and 95 per cent O_2.*

5. *0.5 per cent potassium oxalate solution.* Five grams of pure neutral potassium oxalate are dissolved in distilled water and made up to a liter. Extreme care must be used to obtain oxalate that contains neither carbonate nor free acid. CO_2 analyses should be made on 5 cc. portions of the oxalate solution and on the distilled water used in its preparation. The pH of the oxalate should be approximately 7.4.

6. *Lactic acid, approximately 0.1* N *solution.* Ten cubic centimeters of of concentrated lactic acid of 1.20 specific gravity are diluted to 1 liter.

7. *Sodium hydroxide, 5* N *solution* (see p. 233).

Procedure

Preliminary preparations. Receivers for the blood are prepared by adding 0.2 to 0.3 cc. of the 0.5 per cent potassium oxalate solution to each sealed funnel and evaporating off the water at room temperature.

Sufficient saline phenol red solution for the number of analyses to be run during the day is first brought to pH 7.4.

Collection of sample. Paraffin oil saturated with 5 per cent CO_2—95 per cent O_2 gas mixture is placed in one of the oxalate-containing conical receivers shown in figure 89. A finger is pricked and stuck into the oil, so that the blood collects in the bottom of the receiver without having been exposed to air. If necessary the finger may be gently pressed to accelerate the flow of blood. Six to 12 drops of blood are collected and are stirred thoroughly to dissolve the oxalate and prevent clotting.

Filling the pipettes. The pipettes are filled to the 0.1-cc. mark with the blood. Care must be exercised to prevent oil globules from being drawn up into the pipette with the blood. To prevent the entrance of oil into the pipette while it is inserted through the oil a slight positive air pressure is maintained on the rubber tube at the end of the pipette, so that a little air is being forced out of the pipette tip as the latter passes through the oil. After the pipette tip has entered the blood the latter is drawn up into the capillary until slightly past the 0.1-cc. mark. The pipette is then removed from the funnel and held in a horizontal position while blood is withdrawn from the tip by absorption onto a clean towel until the upper end of the blood column in the capillary falls exactly to the 0.1-cc. mark. Then phenol red saline solution, previously brought to pH 7.4, is drawn up into the pipette until the latter is filled to the 2-cc. mark. Three or 4 pipettes are filled from each sample of blood. In

addition one control pipette is prepared in which the 0.1 cc. of blood is diluted with indicator-free saline soluton instead of the phenol red saline solution.

Determining the volume per cent of cells in the blood. The tip of each pipette is covered with a bit of adhesive tape which is held on by a rubber band as shown in figure 89,*B*, and is centrifuged for one hour at about 2500 revolutions per minute. The per cent of cells is read on the scale of the capillary, as in a hematocrit.

Reading the pH. The pH is determined by the bicolor method of Hastings and Sendroy described on pages 799 to 800. The control pipette prepared with uncolored saline solution is used to balance the yellow color due to the plasma. It is desirable to prepare a control pipette for each plasma, since the depth of color in different plasmas may vary considerably. If the reading is made with the dye-plasma-saline solution at room temperature instead of 38°, the pH at 38° is estimated as described under "pH_s determination on plasma at room temperature with correction," on page 799, for the Hastings-Sendroy method.

Determination of CO_2 in saline blood mixture. 2.5 cc. of 0.1 N lactic acid and 1 drop of caprylic alcohol are extracted for 2 minutes in the chamber of the Van Slyke-Neill manometric apparatus to remove the dissolved gases. The extracted solution, together with about 2 cc. of mercury is run up into the cup above the chamber (see fig. 35, p. 270). The rubber cap is then removed from the pipette and its tip is placed firmly against the bottom of the cup: the pressure of the mercury prevents the solution from emerging from the pipette tip.[8] The stop-cock of the Van Slyke-Neill chamber is then slowly opened and the contents of the pipette are run into the chamber without admission of air (see fig. 52, p. 344). The pipette is rinsed twice by drawing portions of the extracted lactic acid solution up into it and delivering the solution into the chamber. The mercury and the rest of the lactic acid are then run into the chamber. The stop-cock of the latter is sealed with mercury, and the CO_2 is determined as described for micro analyses on page 285 of the gasometric chapter. The manometer reading p_1 is taken with the extracted gases at 0.5-cc. volume, and p_2 is taken after the CO_2 has been absorbed with 0.2 or 0.3 cc. of 5 N sodium hydroxide solution.

[8] If the tip of the pipette has been ground to fit fairly closely the bottom of the cup of the manometric chamber, it will not be necessary to fit a rubber ring about the tip of the pipette. Otherwise it may be necessary to use such a ring, as shown in figures 29 and 30.

A control analysis is performed on 2 cc. of the saline phenol red solution, measured into the chamber of the gas apparatus from an ordinary 2-cc. blood pipette (see figs. 29 and 30 on page 240). The same volumes of distilled water and 1 N lactic acid are used. The value of

TABLE 67

FACTORS FOR CALCULATION OF CO_2 CONTENT OF WHOLE BLOOD IN MICRO ANALYSIS OF SHOCK AND HASTINGS

Factors by which P_{CO_2} is multiplied to calculate the CO_2 content of whole blood analyzed in the micro determination of Shock and Hastings. Sample = 0.1 cc. S = 4.5 cc. a = 0.5 cc. i = 1.037.

TEMPERATURE	FACTORS BY WHICH P_{CO_2} IS MULTIPLIED TO GIVE CO_2 CONTENT OF BLOOD IN:	
°C.	*millimoles per liter*	*vol. per cent*
15	0.320	0.714
16	18	09
17	16	04
18	14	01
19	12	0.698
20	11	92
21	09	89
22	07	85
23	05	81
24	04	77
25	02	73
26	00	70
27	0.299	66
28	97	62
29	96	59
30	94	56
31	92	52
32	90	47
33	89	44
34	87	40

the pressure difference, $p_1 - p_2$ obtained in the control analysis constitutes the c correction for the reagents.

Calculation of the CO_2 content of the blood. The pressure, P_{CO_2}, of the CO_2 extracted from the blood and measured at 0.5-cc. volume is calculated as

$$P_{CO_2} = p_1 - p_2 - c$$

The CO_2 content of the blood is obtained by multiplying P_{CO_2} by the proper factor in table 67.

Data obtainable by the method of Shock and Hastings. Direct determinations are obtained of: 1, the volume per cent of cells in the blood; 2, pH_s at 38°, and, 3, the total CO_2 content of the whole blood. From these data one can obtain: 4, by means of the line chart of figure 39 (p. 287) the CO_2 content of the plasma; and by the chart on figure 41 (p. 294), 5, the CO_2 tension and, 6, $BHCO_3$ content of the plasma.

COLORIMETRIC PLASMA pH. CULLEN'S ORIGINAL METHOD BY COMPARISON WITH PHOSPHATE STANDARDS (6)

The bicolor standards employed in the preceding methods have advantages which ordinarily make their use a procedure of choice for plasma pH. In case, however, the available phenol red proves not sufficiently pure to give correct pH values when compared with standard phosphate solutions, it will be necessary to return to Cullen's original method and compare the color of plasma + saline + dye with the color of phosphate + dye solutions of known pH, given in table 70. It may also be convenient to use this procedure when only a few plasma pH determinations are to be done, and the phosphate standard solutions are already prepared, but the series of bicolor dye standard solutions is not.

Reagents

Saline solution without indicator. 0.9 per cent sodium chloride.

Saline phenol red solution of pH 7.4. To 100 cc. of 0.9 per cent NaCl solution add 35 drops of 0.03 per cent phenol red solution. Then add 0.01 N NaOH solution, as described for the saline phenol red in the Hastings-Sendroy method, until the pH is 7.4. The pH is tested by pouring a portion of the solution into a test tube and comparing the color with that of 15 cc. of a phosphate standard solution of pH 7.4 to which have been added 5 drops of 0.03 per cent phenol red. After the solution has been brought to pH 7.4 it is covered with a layer of paraffin oil. The solution must be made the same day it is used.

0.03 per cent phenol red. Three cubic centimeters of the stock 0.1 per cent solution described in table 60, p. 793, are diluted with water to 10 cc.

Sörensen's standard phosphate solutions. These are prepared for the pH range 7.0 to 7.8 as indicated in table 70. It is essential that KH_2PO_4 and Na_2HPO_4 prepared especially pure for pH standards be used. Merck's preparations made according to Sörensen's directions have proven satis-

factory. If Na_2HPO_4 with water of crystallization instead of the anhydrous salt is used a larger amount must be taken: instead of 9.47 grams of $NaHPO_4$, 11.87 grams of $Na_2HPO_4 \cdot 2H_2O$ are required. The stock solutions, if kept cold in hard glass vessels closed to keep out atmospheric CO_2 (and in some cities SO_2) may, if free from mold, remain unchanged for several weeks.

For the determinations 15-cc. portions of the phosphate solutions are placed in the test tubes, and 5 drops of 0.03 per cent phenol red are added to each. The tubes are kept stoppered. If placed in an ice box when not in use they remain unchanged for two or three days, although even this degree of stability can not be assumed without tests. To be entirely certain of these standards one must make them fresh every day.

Apparatus

The same test tubes and comparator block shown in figure 87 may be used. In the present case, however, two rows of holes suffice in place of the three required when the bicolor standards are used.

Procedure

Twenty cubic centimeters of the saline phenol red solution are placed in one of the test tubes and covered with a layer of paraffin oil; 1 cc. of the plasma is run under the oil into the saline solution. A 1-cc. bulb pipette calibrated to deliver between two marks is more convenient to use than one calibrated for complete delivery, as it is not easy to obtain complete delivery with the pipette tip under the oil layer. Plasma and saline solution are mixed by a minimum of gentle stirring with a rod introduced through the oil.

A control tube is prepared by mixing 20 cc. of the uncolored saline solution with 1 cc. of the same plasma.

The tube of plasma + saline + dye is placed in the comparator, and beside it the standard tube which appears most similar in color. Behind the standard tube is placed the control tube of plasma + uncolored saline solution. The standard tube is changed until a standard is found which matches the plasma + dye tube, or until the color of the latter is located between two of the standards. With standards at intervals of 0.05 pH it is possible to estimate the pH of the plasma solution with a constancy of 0.02 pH, although the error in pathological plasma may be greater because of variations in the correction discussed below.

Calculation of pH_s at 38° from Cullen pH determined at room temperature. Cullen found that normal human plasma handled in the above manner at

20° gave pH values 0.22 unit higher than he found in the same plasma at 38° by the standard hydrogen electrode method. Consequently 0.22 is to be subtracted from plasma pH values obtained at 20° in order to obtain the actual pH_s at 38°.

In case the room temperature was raised above 20°, Cullen found that the correction, between 20° and 30°, decreased by 0.01 pH for each 1° increase in temperature. The correction necessary in order to change pH_s values obtained at room temperature t° to pH_s at 38° is therefore calculated as

$$\text{Correction} = 0.42 - 0.01\ t$$

where t is the temperature centigrade. This correction is *subtracted* from the pH found at room temperature t; e.g., if pH of 7.56 is read at 22°, the correction is $0.42 - 0.22 = 0.20$. The pH_s at 38° therefore is $7.56 - 0.20 = 7.36$.

For plasmas from blood of other species the corrections are different, viz,. at 20°, for the rabbit 0.17; horse, 0.12; dog, 0.35. Bennett (3a) found that in the dog the correction was variable, especially after hemorrhage, the range of variation being from 0.40 to 0.19 pH. Bennett also found that warming dog plasma to 38°, as in the Hastings-Sendroy method, did not completely abolish the correction, although it diminished it.

Although the Cullen average correction of 0.22 pH at 20° holds quite constantly for normal human plasma Austin, Stadie, and Robinson (3) have found that *the correction in some pathological plasmas varies considerably from this figure.* They therefore propose that in such plasmas the correction be determined in each individual case by saturating at 38° a portion of plasma with air containing CO_2 at known tension, preferably about 40 mm., and determining the CO_2 content of the saturated plasma. The technique for saturating plasma with gas of known CO_2 tension is described on page 302 of chapter VII. From the CO_2 tension and the CO_2 content the pH is calculated, either by Hasselbalch's equation (see p. 308) or, more simply, by the line chart in figure 41 (p. 294). On another sample of the same saturated portion of plasma the colorimetric pH is determined with the plasma at room temperature, as above described. The difference between the two pH values is the correction for the plasma of that individual.

The correction can, even more accurately, be determined by comparison of colorimetric pH with pH determined by the hydrogen electrode. In this case a portion of the plasma is saturated at 38° with hydrogen containing about 5.5 per cent of CO_2. In one portion the pH is determined at 38° with the gas electrode, the same hydrogen-CO_2 mixture being used in the

TABLE 68

INDICATORS SELECTED FOR USE IN TITRATION AND THE PREPARATION OF COLOR STANDARDS. DATA TAKEN MOSTLY FROM CLARK (5)

COMMON NAME	RANGE	COLOR CHANGE ACID TO ALKALINE	FORM IN WHICH INDICATOR IS PREPARED FOR USE	0.01 N NaOH PER 0.1 GRAM INDICATOR FOR Na SALT
				cc.
Thymol blue (acid range)	1.2–2.8	Red-yellow	Water solution, Na salt 0.1 per cent	21.5
Tropaeolin OO	1.4–2.6	Red-yellow	Water solution, 0.02 per cent	
Dimethyl amino-azo-benzene	3–4	Red-yellow	Alcohol solution, 0.5 per cent	
Brom phenol blue	3.0–4.6	Yellow-blue	Water solution, Na salt 0.1 per cent	14.9
Methyl orange	3.1–4.4	Red-yellow	0.1 per cent solution in 50 per cent alcohol	
Congo red	4–5	Blue-red	Alcohol solution, 0.5 per cent	
Brom cresol green	3.8–5.8	Yellow-blue	Water solution, Na salt 0.1 per cent	14.3
Methyl red	4.2–6.4	Red-yellow	Alcohol solution, 0.05 per cent	
Alizarin, Clark (5) Index No. 166	5.5–6.8	Yellow-red	Water solution, Na salt 0.1 per cent	
Brom cresol purple	5.2–6.8	Yellow-purple	Water solution, Na salt 0.1 per cent	18.5
Litmus paper	About 7	Red-blue		
Brom-thymol-blue	6.0–7.6	Yellow-blue	Water solution, Na salt 0.1 per cent	16.0
Neutral red	6.8–8.0	Red-yellow	0.1 per cent solution in 50 per cent alcohol	
Phenol red	6.8–8.4	Yellow-red	Water solution, Na salt 0.1 per cent	28.2
Cresol red	7.2–8.8	Yellow-red	Water solution Na salt 0.1 per cent	26.2
Cresolphthalein	8.2–9.8	Colorless-red	Alcohol solution, 0.05 per cent	
Phenolphthalein	8.3–10.0	Colorless-red	1 per cent solution in 50 per cent alcohol	
Thymol blue (alkaline range)	8.0–9.6	Yellow-blue	Water solution, Na salt 0.1 per cent	21.5

electrode. In another portion the colorimetric pH is determined at room temperature or 20° by Cullen's method. The difference is the correction.

This is the technique for determining the correction which was used by Cullen (6).

Urine pH determinations can also be done with acetate and phosphate monocolor standards (tables 69 and 70) in place of the bicolor standards previously described. The corrections required when the determinations are made at room temperature have been given in table 66.

TABLE 69

WALPOLE'S ACETATE BUFFER MIXTURES FOR pH 3.6 TO 5.8

Walpole's 0.2 N acetate buffer mixtures. 0.2 N sodium acetate; 27.22 gm. of sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$ diluted to a liter. 0.2 N acetic acid; approximately 11.3 cc. of 99 per cent glacial acetic acid diluted to a liter. Standardize against 0.1 N sodium hydroxide, with phenolphthalein as indicator. From Walpole (41).

pH	0.2 N SODIUM ACETATE	0.2 N ACETIC ACID
	cc.	*cc.*
3.6	7.5	92.5
3.8	12.0	88.0
4.0	18.0	82.0
4.2	26.5	73.5
4.4	37.0	63.0
4.6	48.0	52.0
4.8	59.0	41.0
5.0	70.0	30.0
5.2	79.0	21.0
5.4	86.0	14.0
5.6	91.0	9.0
5.8	94.0	6.0

DIALYSIS METHOD FOR THE pH OF BLOOD. DALE AND EVANS (10). MODIFICATION OF LEVY, ROWNTREE, AND MARRIOTT'S METHOD (25)

Reagents

0.85 per cent NaCl in freshly boiled redistilled water.

0.02 per cent neutral red solution.

Ten per cent collodion in alcohol-ether.

M/15 solution of Na_2HPO_4 plus 1/50 volume of 0.1 per cent neutral red (see tables 68 and 70).

M/15 solution of KH_2PO_4 plus 1/50 volume of 0.1 per cent neutral red (see tables 68 and 70).

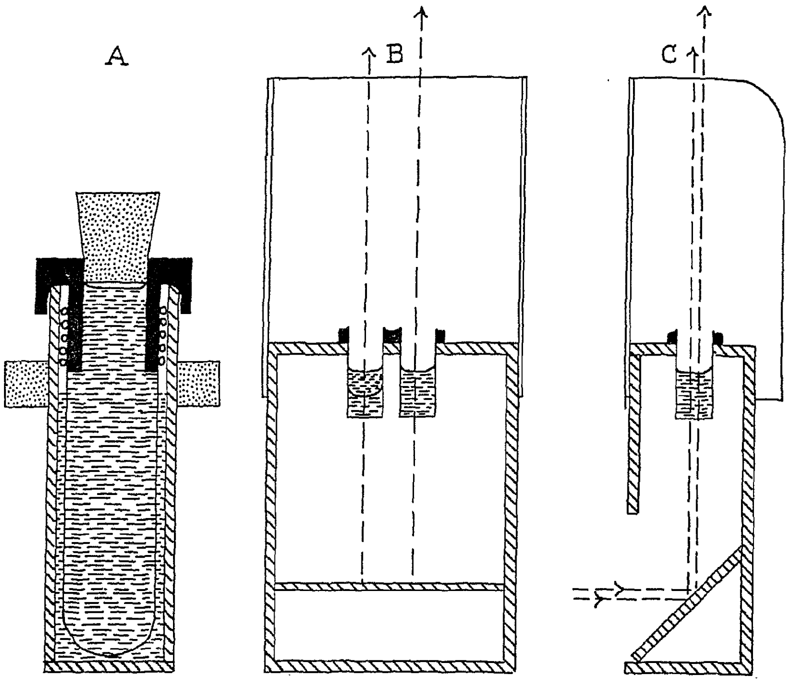


FIG. 90. Section of dialyzer, A, and comparator chamber, B and C, of Dale and Evans (10) for the colorimetric determination of pH, with dialyzer and comparator vessel filled for a determination.

Comparator tubes are made from glass tubing with an external diameter of 14 mm. and an internal diameter of 11.5 mm., cut in pieces 40 mm. long. To the ends, which are ground flat, circular discs, cut from microscope slides of white glass, are cemented with Canada balsam.

Vulcanite membrane holder, turned from a single piece of vulcanite, is shown in solid black. It has an upper orifice that can be closed with a rubber stopper and a groove near the lower end to facilitate attachment of the membrane. Near the upper end is a wide flange, the under surface of which is deeply grooved. The upper and outer surfaces of this groove fit closely the glass tube, while the inner surface remains far enough from the inner surface of the tube to receive the membrane and the ligatures which hold it in place. The dialyzing membrane is made, from 10 per cent collodion in alcohol ether, of such a size that when it is full it occupies all but about 1 cc. of the space in the glass tube.

The comparator is a small box with blackened interior, perforated on top by two holes, placed close together, which receive the comparator vessels. The lower portion of the front wall of the box is open to admit light, which is reflected up through the tubes by a sheet of opal glass placed at an angle of 45 degrees. Around the top of the box is a blackened screen to exclude light from above.

Procedure

The essential apparatus (see fig. 90) consists of a flat-bottomed cylindrical vessel into which a tubular dialyzing membrane fits loosely. The membrane is tied on to the vulcanite holder which has a groove cut round it for this purpose. The vulcanite holder fits closely over the glass vessel by means of the wide thick flange, the under surface of which is cut into a deep angular groove. The finished dialyzing membrane, made from the collodion mixture, is of such size that 1 cc. of fluid nearly fills the space between the sack and the vessel.

One cubic centimeter of 0.85 per cent NaCl solution is placed in the vessel, *A*. The moist membrane sack, attached to its holder, is lowered into the vessel and a pinch of powdered carbonate-free potassium oxalate dropped into it. The sack is steadily filled from below upwards with blood until the vessel is filled, when the stopper is inserted. Dialysis is allowed to proceed for ten to fifteen minutes.

The membrane is removed, and 0.1 cc. of 0.02 per cent neutral red solution is added to the dialysate, which is then covered with paraffin oil to prevent loss of CO_2 and consequent increase of pH. In a tube similar to that containing the dialysate is placed a similar, accurately measured, volume of phosphate-neutral red solution, made up according to table 70 to have about the pH expected in the dialysate. Ordinarily a mixture of 80 parts of Na_2HPO_4 and 20 parts of KH_2PO_4 solution will be used, with a pH of 7.38. The colors in the two tubes are compared. To the standard tube is added, from a micro burette (see figure 1), more of either Na_2HPO_4 + neutral red or KH_2PO_4 + neutral red solution, until the colors in the two tubes match. The tubes are viewed from above, by transmitted light entering from the bottom, as shown in figure 90 C.

When the colors match, the molar percentage of the total phosphate in the form of Na_2HPO_4 is calculated as the ratio,

$$\frac{100 \times \text{cc. } Na_2HPO_4}{\text{cc. } Na_2HPO_4 + \text{cc. } KH_2PO_4}$$

From this value the pH is ascertained by reference to the second column of table 70. If many determinations are to be done, it is convenient to plot the values of this column against pH values, as in figure 42, but on a larger and more accurate scale. The curve thus obtained can conveniently be used for graphic interpolations of pH values from observed phosphate ratios.

TABLE 70

SÖRENSEN'S M/15 PHOSPHATE MIXTURES FOR 5.8 TO 8.2

M/15 primary phosphate, 9.08 grams KH_2PO_4 per liter. M/15 secondary phosphate, 9.47 grams Na_2HPO_4 (anhydrous) per liter. From Sörensen (37).

$pH_{20°}$	$\frac{M}{15}$ Na_2HPO_4	$\frac{M}{15}$ KH_2PO_4
	cc.	*cc.*
5.8	8.0	92.0
5.9	9.0	90.1
6.0	12.2	87.8
6.1	15.3	84.7
6.2	18.6	81.4
6.3	22.4	77.6
6.4	26.7	73.3
6.5	31.8	68.2
6.6	37.5	62.5
6.7	43.5	56.5
6.8	49.6	50.4
6.85	52.5	47.5
6.90	55.4	44.6
6.95	58.2	41.8
7.00	61.1	38.9
7.05	63.9	36.1
7.10	66.6	33.4
7.15	69.2	30.8
7.20	72.0	28.0
7.25	74.4	25.6
7.30	76.8	23.2
7.35	78.9	21.1
7.40	80.8	19.2
7.45	82.5	17.5
7.50	84.1	15.9
7.55	85.7	14.3
7.60	87.0	13.0
7.65	88.2	11.8
7.70	89.4	10.6
7.75	90.5	9.5
7.80	91.5	8.5
7.90	93.2	6.8
8.0	94.7	5.3
8.1	95.8	4.2
8.2	97.0	3.0

Subtract 0.03 for pH at 38°.

Lindhard (26) for determining the pH of the dialysate, reverted to the usual colorimetric pH technique, which had been employed also by Levy,

Rowntree, and Marriott (25), using phenol red as indicator, viewing the tubes from the side, and comparing the dialysate with a series of phosphate standards prepared according to table 70.

Remarks

Besides the observational error and the error that may be involved in making the reading with the solutions at room temperature instead of body temperature, Van Slyke, Wu and McLean (40) have calculated that when serum (or blood) is dialyzed against a solution of approximately the same

TABLE 71

CLARK'S (5) 0.2 M BORATE BUFFER MIXTURES FOR pH 7.8 TO 10

0.2 M boric acid and 0.2 M potassium chloride. 12.405 grams H_3BO_3 + 14.912 grams KCl per liter. 0.2 M sodium hydroxide, from concentrated CO_2 free alkali (5).

pH	0.2 M H_3BO_3 + 0.2 M KCl	0.2 M NaOH
	cc.	*cc.*
7.8	50	2.65
8.0	50	4.00
8.2	50	5.90
8.4	50	8.55
8.6	50	12.00
8.8	50	16.40
9.0	50	21.40
9.2	50	26.70
9.4	50	32.00
9.6	50	36.85
9.8	50	40.80
10.0	50	43.90

Dilute to 200 cc. with water in each case.

salt concentration, one may expect, as a result of the Donnan effect, a pH in the salt solution about 0.02 greater than in the serum. A similar correction was estimated by Taylor (39) from the potential difference between the fluid in the sac and the saline solution outside. One should therefore subtract 0.02 from the pH reading obtained in the dialysate.

GASOMETRIC PLASMA pH DETERMINATION

The principle of these procedures has been outlined in the introduction to this chapter. The techniques of the method of Eisenman, based on interpolation of the CO_2 content of serum on the serum CO_2 absorption curve, and of the method of Van Slyke, Sendroy, and Liu, based on estima-

tion of the CO_2 tension in a bubble of air equilibrated with a sample of fresh whole blood, are described on pages 298 and 309 of chapter VII.

BIBLIOGRAPHY

1. Austin, J. H., and Cullen, G. E.: Hydrogen ion concentration of the blood in health and disease. Medicine Monographs. Baltimore, 1926, Williams and Wilkins.
2. Austin, J. H., Cullen, G. E.; Hastings, A. B.; McLean, F. C.; Peters, J. P., and Van Slyke, D. D.: Studies of gas and electrolyte equilibria in blood. I. Technique for collection and analysis of blood, and for its saturation with gas mixtures of known composition. *J. Biol. Chem.*, 1922, **54**, 121.
3. Austin, J. H., Stadie, W. C., and Robinson, H. W.: The relation between colorimetric reading and true pH of serum or plasma. *J. Biol. Chem.*, 1925, **66**, 505.

3a. Bennett, Mary A.: Cullen's colorimetric method for determination of the pH of blood plasma. *Proc. Soc. Exp. Biol. Med.*, 1925, **23**, 115.

4. Bjerrum, N.: Die Theorie der alkalimetrischen und acidimetrischen Titrierungen, 1914, Stuttgart.
5. Clark, W. M.: The determination of hydrogen ions. 3rd Ed., 1928, Baltimore, Williams and Wilkins.
6. Cullen, G. E.: Studies of acidosis. XIX. The colorimetric determination of the hydrogen ion concentration of blood plasma. *J. Biol. Chem.*, 1922, **52**, 501. A modification of the Clark hydrogen electrode vessel to permit accurate temperature control. *J. Biol. Chem.*, 1922, **52**, 521.
7. Cullen, G. E.: An improved form of the quinhydrone electrode. *J. Biol. Chem.*, 1929, **83**, 535.
8. Cullen, G. E., and Biilmann, E.: The use of the quinhydrone electrode for hydrion concentration determination on serum. *J. Biol. Chem.*, 1925, **64**, 727.
9. Cullen, G. E., and Earle, I. P.: On the determination of the pH of blood. I. The accuracy of the quinhydrone electrode for determining the pH of blood plasma or serum. *J. Biol. Chem.*, 1928, **76**, 565.
10. Dale, H. H., and Evans, C. L.: Colorimetric determination of the reaction of blood by dialysis. *J. Physiol.*, 1920–21, **54**, 167.
11. Davis, J. E., and Hastings, A. B.: Personal communication.
12. Earle, I. P., and Cullen, G. E.: On the determination of the pH of the blood. II. A comparison of the colorimetric method with the hydrogen and quinhydrone electrodes. *J. Biol. Chem.*, 1928, **76**, 583.
13. Eisenman, A. J.: A gasometric method for the determination of pH in blood. *J. Biol. Chem.*, 1927, **71**, 611.
14. Gillespie, L. J.: Colorimetric determination of hydrogen ion concentration without buffer mixtures. *J. Am. Chem. Soc.*, 1920, **42**, 742.
15. Hasselbalch, K. A.: Die Berechnung der Wasserstoffzahl des Blutes aus der freien und gebundenen Kohlensäure desselben, und die Saurestoffbindung des Blutes als Funktion der Wasserstoffzahl. *Biochem. Z.*, 1117, **78**, 112.
16. Hasselbalch, K. A., and Lundsgaard, C.: Elektrometrische Reaktionsbestimmung des Blutes bei Körpertemperatur. *Biochem. Z.*, 1912, **38**, 77.
17. Hastings, A. B., and Sendroy, J. Jr.: Studies of acidosis. XX. The colorimetric determination of blood pH at body temperature without buffer standards. *J. Biol. Chem.*, 1924, **61**, 695.

18. HASTINGS, A. B., SENDROY, J., JR., AND ROBSON, W.: Studies of acidosis. XXI. The colorimetric determination of the pH of urine. *J. Biol. Chem.*, 1925, **65**, 381.
19. HAVARD, R. E., AND KERRIDGE, P. T.: An immediate acid change in shed blood. *Biochem. J.*, 1929, **23**, 600.
20. HAWKINS, J. A.: A micro method for the determination of the hydrogen ion concentration of whole blood. *J. Biol. Chem.*, 1923, **57**, 493.
21. HENDERSON, L. J., AND PALMER, W. W.: On the intensity of urinary acidity in normal and pathological conditions. *J. Biol. Chem.*, 1913, **13**, 393.
22. HÖBER, R.: Ueber die Hydroxylionen des Blutes. *Pflügers Arch. Physiol.*, 1900, **81**, 522.
II. *Ibid.*, 1903, **99**, 572.
23. KERRIDGE, P. T.: The use of the glass electrode in biochemistry. *Biochem. J.*, 1923, **19**, 611.
24. LAUG, E. P.: The application of the quinhydrone electrode to the determination of the pH of serum and plasma. *J. Biol. Chem.*, 1930, **88**, 551.
25. LEVY, R. L., ROWNTREE, L. G., AND MARRIOTT, W. MCK.: A simple method for determining variations in the hydrogen-ion concentration of the blood. *Arch. Int. Med.*, 1915, **16**, 389.
26. LINDHARD, J.: Colorimetric determination of the concentration of hydrogen ions in very small quantities of blood by dialysis. *Compt. rend. trav. lab. Carlsberg*, 1921, **14**, [13] 13.
27. MACINNES, D. A., AND DOLE, M.: A glass electrode apparatus for measuring the, pH values of very small volumes of solution. *J. Gen. Physiol.*, 1929, **21**, 805.
28. MICHAELIS, L., AND DAVIDOFF, W., Methodisches und Sachliches zur elektrometrischen Bestimmung der Blutalkalescenz. *Biochem. Z.*, 1912, **46**, 131.
29. MYERS, V. C.: A colorimeter for bicolorimetric work. *J. Biol. Chem.*, 1922, **54**, 675.
30. MYERS, V. C., AND MUNTWYLER, E.: Colorimetric estimation of the hydrogen ion concentration of the urine. *J. Biol. Chem.*, 1928, **78**, 225.
31. MYERS, V. C., AND MUNTWYLER, E.: The colorimetric estimation of the hydrogen ion concentration of blood. *J. Biol. Chem.*, 1928, **78**, 243.
32. MYERS, V. C., SCHMITZ, H. W., AND BOOHER, L. E.: A micro colorimetric method for estimating the hydrogen ion concentration of the blood. *J. Biol. Chem.* 1923, **57**, 209.
33. PARSONS, T. R.: On the reaction of the blood in the body. *J. Physiol.*, 1917, **51**, 440.
34. SENDROY, J., JR., AND HASTINGS, A. B.: The activity coefficients of certain acid-base indicators. *J. Biol. Chem.*, 1929, **82**, 197.
35. SHOCK, N. W., AND HASTINGS, A. B.: A micro technique for the determination of the acid-base balance of the blood. *Proc. Soc. Exp. Biol. Med.*, 1929, **26**, 780.
36. SHOHL, A. T.: The determination of pH and carbon dioxide on a single small sample of blood plasma or serum. *J. Biol. Chem.*, 1929, **83**, 759.
37. SÖRENSEN, S. P. L.: Über die Messung und Bedeutung der Wasserstoffionenkonzentration bei biologischen Prozessen. *Ergebn. Physiol.*, 1912, **12**, 393.
38. STADIE, W. C., O'BRIEN, H., AND LAUG, E. P.: Determination of the pH of serum at 38° with the glass electrode and an improved electron tube potentiometer. *J. Biol. Chem.*, 1931, **91**, 243.

39. Taylor, H.: The accuracy of the Dale and Evans method of determining the H-ion concentration of the blood. *Biochem. J.*, 1923, **17**, 406.

40. Van Slyke, D. D., Wu, H., and McLean, F. C.: Studies of gas and electrolyte equilibria in the blood. V. Factors controlling the electrolyte and water distribution in the blood. *J. Biol. Chem.*, 1923, **56**, 765.

41. Walpole, G. C.: Hydrogen potentials of mixtures of acetic acid and sodium acetate. *J. Chem. Soc.*, 1914, **105**, 2501.

42. Warburg, E. L.: Studies on carbonic acid compounds and hydrogen ion activities in blood and salt solutions. *Biochem. J.*, 1922, **16**, 153.

43. Wu, H.: A modification of the Duboscq-Pellin colorimeter for bi-colorimetric work, as in colorimetric pH determination without buffer mixtures. *Proc. Soc. Exp. Biol. Med.*, 1923, **21**, 111.

CHAPTER XXVIII

BICARBONATE

DISCUSSION

Of the various analytical procedures for determining bicarbonate, two have proven their practical applicability to physiological fluids.

The *gasometric* method is probably the one which is at present in most general use. The total CO_2 content of blood or urine is determined, and from it the fraction present as $BHCO_3$ is calculated, either from the pH by the Henderson-Hasselbalch formula, or by subtracting the H_2CO_3 estimated from the observed CO_2 tension. These procedures have been described on page 292 of chapter VII.

For *titration* of bicarbonate the procedure used in ordinary inorganic analysis is to add standard hydrochloric or sulfuric acid until an end-point is reached with an indicator like methyl orange, at a pH below 4, where the acidity is so high that all quantitatively significant portions of the CO_2 are in the form of free carbonic acid. When buffers other than bicarbonate are present in significant amounts, however, this method can not be used, because the other buffers would neutralize part of the added acid. In most physiological fluids such buffers are present, notably the phosphates and salts of organic acids in urine, and the proteins in blood plasma.

In order to titrate accurately the bicarbonate of such fluids it is necessary, as a net result of the operation, to transform the $BHCO_3$ into the salt of the acid used in the titration, and to have as the end-point the same pH originally present in the fluid. If the final pH is lower, the alkali of buffers other than $BHCO_3$ will neutralize part of the acid added in the titration, and the $BHCO_3$ will be calculated too high.

The necessary conditions have been met by Van Slyke, Stillman, and Cullen (3), who added an excess of 0.02 N HCl to plasma, aerated to remove all the CO_2, and then titrated back with 0.02 N NaOH to pH 7.4. The procedure was later improved (2) by using as end-point the pH of a control portion of the same plasma, so that protein errors of the indicator were compensated, and the original pH of the particular plasma analyzed became the end-point in each case.

The titration is not quite so precise as the gasometric method, particu-

larly when the latter is carried out with the manometric apparatus. The titration, however, seldom differs by more than 0.3 millimole of $BHCO_3$ per liter (= 0.7 volume per cent of CO_2) from the gasometric result, and is entirely adequate for most studies of the acid base balance.

Dilution and acidification of plasma are performed with solutions containing 0.9 per cent of NaCl, in order to keep globulin in solution at the end-point.

TITRATION OF BICARBONATE IN SERUM OR PLASMA

Reagents

Phenol red, 0.03 per cent solution of the sodium salt prepared as directed in the pH chapter (p. 793).

Saline phenol red solution of pH 7.4. As described on p. 809.

Hydrochloric acid, 0.01 N made by diluting 1 volume of 0.1 N hydrochloric acid to 10 volumes with neutral CO_2-free 1 per cent sodium chloride solution.

Sodium hydroxide, 0.01 N CO_2-free, prepared from CO_2-free 0.01 N sodium hydroxide, by dilution with neutral, CO_2-free, 1 per cent sodium chloride solution. (For the preparation and preservation of CO_2-free alkali, see chapter I, page 29. Observe also precautions for "Preparation and preservation of standard solutions of sodium hydroxide more dilute than 0.1 N" on page 30.)

The standard 0.01 N sodium hydroxide should be made up the day it is used, and should be tested for the presence of carbonate as follows (1).

To 5 cc. of the 0.01 N HCl in a 200-cc. round-bottomed flask add from a freshly filled burette about 4.8 cc. of the 0.01N NaOH, and 0.3 cc. of the phenol red solution. The mixture should be strongly acid to the indicator. The solution is whirled about the walls of the flask for one minute to permit the escape of any CO_2 that may have been present in the standard alkali. The solution is then transferred to a 50-cc. test tube or flask, with the use of 10 cc. of the neutral sodium chloride solution to wash the 200-cc. flask. Enough additional 0.01 N NaOH is then added from the burette to bring the solution to pH 7.4. The end-point is controlled by comparison with 20 cc. of Soerensen's phosphate solution (table 70, p. 816) of pH 7.4.

A duplicate titration is performed in the same way, except that there is no agitation to remove carbon dioxide, the 0.01 N HCl plus 10 cc. of neutral NaCl solution being placed directly in the 50-cc. flask or tube, and the 0.01 N NaOH being added with a minimum of stirring.

If there were no carbonate in the standard NaOH solution the two titrations would give identical results. The difference should preferably not exceed 0.1 cc., and if it exceeds 0.3 cc. the alkali should not be used.

Procedure

From blood drawn and centrifuged with rigorous precautions to prevent loss of CO_2 (see chapter II), serum or plasma is secured; 1 cc. of this plasma is transferred with similar precautions to a tube, 20-mm. in diameter, containing, under oil, 20 cc. of saline phenol red solution of pH 7.4. This tube serves as a standard for the end-point.

To another 1 cc. sample, in a round-bottomed flask of about 200-cc. capacity, are added 5 cc. of 0.01 N hydrochloric acid in 0.9 per cent sodium chloride. The flask is whirled about for at least one minute to facilitate escape of CO_2 from the acidified solution. The solution is then poured into a test-tube of the same diameter as the one containing the standard, the flask being rinsed with 10 cc. of neutral 0.9 per cent sodium chloride. Seven drops of 0.03 per cent phenol red are added and 0.01 N sodium hydroxide in salt solution is run in from a burette (fig. 1, p. 13) which permits readings to 0.01 cc., until the color of the solution matches that of the standard. The cubic centimeters of 0.01 N alkali used is designated as ***A***.

As the end-point is approached, each drop of alkali added appears to change the color past the end-point. Within a few seconds the color shifts back, and it is seen that at least another drop is needed before the genuine end-point is reached. Consequently the final color comparison should not be made until at least thirty seconds after the last drop of alkali has been added. Because of this behavior, it is well to overrun the end-point by a drop of alkali, rather than to stop short of it when in doubt (3).

A blank titration is done, in which 5 cc. of the same 0.01 N acid are diluted with 10 cc. of the neutral 0.9 per cent NaCl solution and titrated with the same 0.01 N alkali to the pH of the serum (or to pH 7.4 with a control tube of phosphate buffer (see table 70, p. 816)). The number of cubic centimeters of 0.01 N alkali required for the blank is designated as ***B***.

Calculation

$$10\ (B - A) = \text{millimoles of } BHCO_3 \text{ per liter of plasma.}$$

$$22.3\ (B - A) = \text{volumes per cent of } CO_2 \text{ in the form of } BHCO_3.$$

A = cubic centimeters of 0.01 N NaOH used in titrating the plasma, B = cubic centimeters of 0.01 N NaOH used in the blank titration.

BIBLIOGRAPHY

1. Stillman, Edgar: Studies of acidosis. XIV. Determinations of bicarbonate in the blood plasma of different species by the titration and carbon dioxide capacity methods. *J. Biol. Chem.*, 1919, **39**, 261.
2. Van Slyke, D. D.: Studies of acidosis. XVIII. Determination of the bicarbonate concentration of the blood and plasma. *J. Biol. Chem.*, 1922, **52**, 495.
3. Van Slyke, D. D.; Stillman, Edgar, and Cullen, G. E.: Studies of acidosis. XIII. A method for titrating the plasma bicarbonate content of the plasma. *J. Biol. Chem.*, 1919, **38**, 167.

CHAPTER XXIX

Titratable Acid and Acid-base Excretion in Urine

BASE ECONOMY

The term "base economy" has been introduced by Gamble (2), to indicate the amount of bicarbonate loss which is spared the body by the excretion of acid radicals in excess of fixed base (fixed base = base other than NH_4 = $Na + K + Ca + Mg$). The base economy may be calculated in two ways.

A. From the ammonia, titratable acid, and bicarbonate excretion. Excretion of acid radicals in the form of neutral salts of fixed bases (e.g., as NaCl of Na_2SO_4) does not contribute to the maintenance of the alkali reserve of the organism, because the acid radicals take out with them equivalent amounts of Na, K, Ca or Mg. It is only excretion of acids either free or as ammonium salts that contributes to the base economy of the organism. The sum of the free, titratable acids and the ammonia of the urine therefore is a measure of the base economy. In alkaline urine there is no free titratable acid. In place of it there may be large amounts of alkali bicarbonate. This constitutes a direct loss of alkali reserve from the body, and therefore must be subtracted from the ammonia + titratable acid to give the base economy of the urinary excretion. Therefore

$$\text{Base economy} = \text{Ammonia} + \text{Titratable acid} - \text{Bicarbonate.}$$

In this case the titratable acid represents acid titrated by Henderson and Palmer's (4) method with the pH of the blood plasma, 7.4, as end-point.

If excretion of bicarbonate exceeds that of ammonia + titratable acid, there is loss of alkali from the body, and the base economy is negative.

B. From the difference between fixed alkali and acid radicals excreted. This calculation simply involves adding the number of monovalent equivalents of bases, other than ammonia, and of acid radicals, other than carbonic, and determining the excess of such acid over basic radicals excreted. The calculation then becomes:

$$\text{Base economy} = (Cl + SO_4 + PO_4 + \text{Organic acids}) - (Na + K + Ca + Mg).$$

all values being expressed in monovalent equivalents, or, as is usually more convenient, milli-equivalents. The monovalent equivalents of SO_4, Ca, and Mg are twice the moles of each. In the calculation formulae given in this

volume, the monovalent equivalent for each of these radicals is obtained directly. For PO_4 the monovalent base-binding equivalent at blood plasma pH is obtained by multiplying the *molar* concentration of PO_4 by 1.8, since at pH 7.4 each molecule of phosphoric acid has one H completely replaced by base and 0.8 of a second H (see fig. 42, p. 295).

If the principles of the above two methods of calculating the base economy of the urinary excretion are correct, and include all the quantitatively important radicals, the values for base economy obtained by the two methods should be approximately equal. Gamble, Tisdall, and Ross (2) have in fact found that the two procedures for calculating the base economy give essentially the same results.

Presumably method *B* can be applied also to fecal excretion. There is no method for determining organic acids in feces, but it is probable that their amount is not great compared with that of the other radicals.

In other chapters we have described the methods for determining all the values involved in calculation of the base economy, either procedure *A* or *B*, with the exception of the titratable acid. We shall accordingly here describe Henderson and Palmer's method for determining this value, by titrating the urine to the end-point of normal plasma, pH 7.4.

In addition we describe Folin's method of titrating to the end-point of phenolphthalein, about pH 8.2. Titration to this more alkaline end-point indicates more free acid, usually by about 25 per cent, than Henderson and Palmer's titration. The Folin titration can not be used like the Henderson-Palmer in determining the base economy of the urinary excretion; but the Folin procedure is somewhat simpler in that it can be carried out without a standard pH solution for comparison in judging the end-point, and the sum of ammonia + Folin titratable acid in diabetic and similar acidoses serves as a simple measure of the intensity of the condition (see columns 3 and 4 of table 63 on p. 1001 of volume I). For these reasons the Folin titration also will be described.

NATURE OF THE TITRATABLE ACID IN URINE

The chief constituent of the titratable acid in normal urine is apparently acid phosphate (3, 4). The lower the pH of the urine the greater is the proportion of phosphate in the form of BH_2PO_4 and therefore the greater the proportion which must be titrated with alkali in order to bring 80 per cent to the form of B_2HPO_4 (for quantitative relations see figure 42). There are also organic buffer acids which occur in urine, exist partly free at pH as low as 5, and are titrated, requiring per mole a fraction of an equivalent of alkali depending upon the initial pH of the urine. Of such acids, how-

ever, the only one that has been demonstrated in important amounts is the beta-hydroxybutyric acid formed in diabetes and other conditions causing ketosis. From the apparent dissociation constant, 2×10^{-5}, (5) one calculates that at pH 4.8, the most acid reaction encountered in urine, 55 per cent of this acid is free, while at pH 7.4 practically all is neutralized. Therefore in a urine of pH 4.8 one would titrate 55 per cent of the beta-hydroxybutyric acid present by the Henderson-Palmer method, and the same proportion by Folin's method.

DETERMINATION OF THE TITRATABLE ACIDITY OF THE URINE BY METHOD OF HENDERSON AND PALMER (4)

Reagents

Phosphate solution. pH 7.4. A M/15 phosphate buffer solution of pH 7.4 prepared as indicated in table 70, page 816.

Sodium hydroxide, 0.1 N.

Phenol red. 0.1 per cent water solution of the sodium salt, prepared as described in the chapter on determination of pH (see table 60), or *neutral red,* 2 per cent water solution. (Neutral red was used by Henderson and Palmer, but the stock phenol red solution can also be employed.)

Procedure

To 10 cc. of the phosphate solution, in a 500-cc. flask, diluted to 250 cc. with water, are added 2 cc. of 0.1 per cent solution of phenol red, or 0.2 cc. of 2 per cent neutral red solution. In a similar flask 10 cc. of urine are treated in the same manner. 0.1 N sodium hydroxide is then added to the diluted urine until its color matches that of the phosphate standard.

Calculation

$10\,A$ = milli-equivalents of titratable acid (cubic centimeters of 1 N acid) per liter of urine.

A = cubic centimeters of alkali used in the titration.

DETERMINATION OF THE TOTAL ACIDITY OF URINE BY THE METHOD OF FOLIN (1)

The urine is first treated with potassium oxalate to eliminate the confusing effects of calcium in combination with phosphoric acid, and is then titrated with sodium hydroxide, with phenolphthalein as indicator.

Reagents

Potassium oxalate, neutral, powdered.
Phenolphthalein, 0.5 per cent in 50 per cent alcohol.
Sodium hydroxide, 0.1 N.

Procedure

To 25 cc. of urine, in a 200-cc. Erlenmeyer flask, add 1 cc. of phenolphthalein indicator and 5 grams of powdered oxalate. Shake the mixture for about one minute and then titrate at once with 0.1 N sodium hydroxide until a distinct pink coloration is produced. The flask should be shaken during the titration to keep the concentration of oxalate in the solution as great as possible.

Calculation

$4A$ = milli-equivalents of titratable acid (cubic centimeters of 1 N) per liter of urine.

A = cubic centimeters of 0.1 N sodium hydroxide used in the titration.

BIBLIOGRAPHY

1. Folin, O.: The acidity of urine. *Am. J. Physiol.*, 1903, **9**, 25.
2. Gamble, J. L.; Ross, G. S., and Tisdall, F. F.: The metabolism of fixed base during fasting. *J. Biol. Chem.*, 1923, **57**, 633.
3. Henderson, L. J.: A critical study of the process of acid excretion. *J. Biol. Chem.*, 1911, **9**, 403.
4. Henderson, L. J., and Palmer, W. W.: On the several factors of acid excretion. *J. Biol. Chem.*, 1914, **17**, 305.
5. Henderson, L. J.; and Spiro, K.: On ionic equilibria in the anomal organism. I. The ionization constants of beta-oxybutyric and acetoacetic acids. *J. Biol. Chem.* 1909, **6**, p. xxxix.

CHAPTER XXX

CHLORIDE

DISCUSSION

Practically all chloride analyses in biological material are now carried out by titration methods. In clinical and physiological work the classical gravimetric procedure of precipitating and weighing as AgCl has been abandoned because it requires so much more time, material, and attention to detail than the special titration procedures that have been developed, with errors less than 1 per cent. Such accuracy is necessary for analyses of plasma chlorides, because the normal and many pathological variations are within a zone of a few per cent. Satisfactory gasometric chloride methods have not been developed, nor sufficiently exact colorimetric ones.

Mohr's titration with chromate indicator. Of the titration methods the oldest and simplest is apparently that of Mohr (15). The chloride is precipitated in neutral or slightly acid solution with standard silver nitrate solution added from a burette, a few drops of potassium or sodium chromate being used as indicator. AgCl and Ag_2CrO_4 are both insoluble, but AgCl is so much the more so that no permanent precipitate of Ag_2CrO_4 is formed until all the Cl has been precipitated. Then the first drop of excess Ag causes formation of a permanent brown cloud of silver chromate precipitate. The chief drawback of this method for biochemical work is that the pH of the titrated solution can not be lower than 5 (3); or the Ag_2CrO_4 precipitate does not form to indicate the end-point. In urine and blood filtrates organic substances, such as the purines, may occur, which are precipitated by silver unless free nitric acid is present in considerable concentration. Consequently the Mohr method is seldom used in analyses of biological material.

Volhard's titration, with sulfocyanate indicator (25). The chloride methods most used in biological analyses are applications of Volhard's method. The Cl is first precipitated by adding an excess of standard $AgNO_3$ solution. Then a ferric salt is added as indicator and standard sulfocyanate is run in from a second burette. A white precipitate of AgSCN is formed until SCN^- in excess of the Ag^+ has been added. Than at once the intense red $Fe(SCN)_3$ forms, and serves as the end-point. This end-point is sensitive even in the presence of free nitric acid.

The reactions are the following:

(1) $Cl^- + Ag^+ = AgCl$ (white precipitate).

(2) Ag^+ (excess from (1)) $+ SCN^- = AgSCN$ (white precipitate).

(3) $3\ SCN^-$ (first drop excess from (2)) $+ Fe^{+++} = Fe(SCN)_3$ (red solution).

Calculation: $Cl = Ag - SCN$.

For a permanent end-point it is necessary after reaction 1 to filter off the AgCl and perform in the filtrate the back titration with sulfocyanate. AgSCN is much more insoluble than AgCl, so that as soon as SCN is present in solution, as $Fe(SCN)_3$, reaction occurs with the previously precipitated AgCl.

(4) $Fe(SCN)_3 + 3\ AgCl = FeCl_3 + 3\ AgSCN$

In consequence of reaction 4 the end-point color formed by 3 fades away. If more sulfocyanate is added a new, but erroneous end-point is obtained, which also fades, so that it is possible to make an error of several per cent if the final titration is performed without removing the AgCl.

Reaction 4 with the precipitated AgCl is less rapid as the AgCl precipitate is more completely coagulated, and as its solubility is diminished by addition of other reagents. Various expedients have been adopted to retard the reaction so that, without filtering off the AgCl, the titration could be carried out in its presence, and the end-point still last long enough to afford an accurate result. Rothmund and Burgstaller (18) added ether after reaction 1 and shook in order to coagulate the AgCl. McLean and Van Slyke (14) obtained the same effect with a drop or two of caprylic alcohol. Claudius (2) added alcohol to diminish the solubility of the AgCl; Smirk (20) and Patterson (17) added acetone with the same effect. Alcohol and acetone also have the advantage that they increase the intensity of the ferric sulfocyanate color by diminishing the dissociation of the salt (the undissociated $Fe(SCN)_3$ is the colored compound). Harvey (11) and Whitehorn (27) increased the permanency of the end-point by optimum regulation of the nitric acid and ferric indicator concentrations.

The admissibility of much free nitric acid at the end-point makes it simple to carry out in a single vessel wet HNO_3 oxidation of proteins and other organic material, and subsequent Volhard titration of the Cl. The $AgNO_3$ is added before the oxidation, because otherwise the chloride would be oxidized to elementary Cl_2 and lost by volatilization. This procedure is used in the classical Carius method for halogen in organic compounds, the ashing being carried out at oven temperature in a bomb tube. In biological

material it has been found that the oxidation can be carried out with boiling nitric acid in unsealed vessels without loss of chlorine; this procedure has therefore been called the "open Carius." In analysis of albuminous material it has the advantage of avoiding necessity for removal of protein by precipitation or dry ashing. The open Carius method for chlorides was first applied in 1897 independently to albuminous urine by Korányi (12) and to tissues and blood by Moraczewski (16). Twenty years later Laudat (13) revived the procedure for blood analyses. These authors, in order to avoid the error due to fading of the end-point from reaction 4, filtered the AgCl precipitate, and titrated the filtrate. Van Slyke and Sendroy (24) avoided the filtration by utilizing end-point conditions, with respect to concentrations of ferric salt and nitric acid, that had been devised by Whitehorn (27) to stabilize the end-point. Claudius (2) and Patterson (17) achieved semi-permanent end-points by adding to the digest alcohol and acetone, respectively; while Fiske and Sokhey (5) removed the AgCl from the reacting fluid by centrifugating the AgCl and AgSCN precipitate mixture just before the end-point was reached, and adding the last drops of sulfocyanate to the supernatant fluid.

In Van Slyke and Sendroy's (24) application of the procedure to blood, the silver nitrate and nitric acid were combined in a single solution, which therefore served both for digestion of proteins and precipitation of chlorides. Wilson and Ball (28) found that sometimes this procedure gave low results. When, however, Wilson and Ball first added to blood silver nitrate separately in aqueous solution, and introduced the nitric acid only after the serum and silver nitrate had been mixed, as had been done by Laudat (13), chloride recoveries were uniformly complete. Eisenman (4), who had employed the Van Slyke-Sendroy method for several years in the laboratory of one of the authors, encountered none of Wilson and Ball's difficulties, and in a series of checks secured consistent agreement between the original Van Slyke-Sendroy technique and the Wilson and Ball modification. She pointed out that it is important to add the silver nitrate-nitric acid mixture to the serum *slowly and with constant stirring.* Her results indicate that the original Van Slyke-Sendroy (24) procedure, if carried out with this precaution, gives uniformly consistent and correct values. It has, over the Wilson-Ball modification, two advantages:

1. Instead of measuring, for analysis of 1 cc. of serum, 1 cc. of standard aqueous 0.15 N $AgNO_3$ solution, one measures 3 cc. of 0.05 N $AgNO_3$ in HNO_3: accuracy is easier with the larger volume.

2. The HNO_3-$AgNO_3$ reagent of Van Slyke and Sendroy keeps indefinitely, while the 0.15 N aqueous silver nitrate used in the Wilson and

Ball modification may in time weaken by reduction and precipitation of some of the silver in the metallic form.

Nitric acid digestion of whole blood and, to a lesser extent, of serum yields solutions with sufficient yellow color to make it difficult for some analysts to detect with maximum accuracy the end-point of the titration. The color can be destroyed by the addition of a little potassium permanganate during the digestion.

McLean and Van Slyke's iodometric titration with nitrite-starch indicator (*14*). McLean and Van Slyke in 1915 developed a titration with an end-point even more sensitive than that of Volhard. As in the Volhard analysis, the Cl is precipitated with an excess of Ag, but the excess of Ag is titrated back with standard KI solution instead of sulfocyanate; the end-point is formed by the iodine which is set free by reaction of nitrous acid with the first drop of unprecipitated KI solution. The reactions are:

(1) $$Cl^- + Ag^+ = AgCl \text{ (white precipitate).}$$

(2) $$Ag^+ \text{ (excess from reaction 1) } + I^- = AgI \text{ (yellow precipitate).}$$

(3) $$HI \text{ (first drop excess from 2) } + HNO_2 = I + NO + H_2O \text{ (blue color of iodine with starch).}$$

Calculation: $Cl = Ag - I$.

The sensitivity of the end-point is such that the color made by 1 drop of 0.01 N iodide can be seen in 75 cc. of solution.

As in the original Volhard titration, it is necessary to filter off the AgCl before the back titration with KI solution. Since AgI, like AgSCN, is much more insoluble than AgCl, traces of soluble iodide or iodine in the presence of AgCl rapidly disappear from solution by reaction 4.

(4) $$I^- + AgCl = Cl^- + AgI.$$

The difference in solubility between AgI and AgCl is, in fact, so marked that no devices have been offered which render possible the omission of filtration of the AgCl before the final titration.

Despite this fact, the sensitivity and permanence of the end-point offer such an attraction that the procedure has been preferred in a number of methods for titrating blood filtrates (1, 6, 7, 9, 10, 19, 21). It has not yet been applied to nitric acid digests, which contain so much free nitric acid that rather large amounts of buffer would be required to bring the final reaction to the optimum pH for the iodine end-point.

Choice of methods

For urine we describe only the Harvey application of the Volhard titration, which apparently meets all requirements with maximum simplicity.

For direct analyses of blood and plasma, and of feces, modifications of the open Carius method, with combined nitric acid ashing and Volhard titration are outlined with modifications for 1, 0.2, and 0.02 cc. of plasma.

For analyses of the Folin-Wu blood filtrate, Whitehorn's application of the Volhard titration and an application of the McLean-Van Slyke iodometric titration are given.

When preparation of Folin-Wu blood filtrates is not required for other analyses, the method of choice for plasma chlorides will ordinarily be one of open Carius procedures, in which the entire analysis is completed in one vessel. When the Folin-Wu filtrate is prepared for other analyses, it may be more convenient to use it for chlorides also. In this case the analyst can follow his individual preference for the Volhard titration, as applied by Whitehorn, or for the iodometric titration of McLean and Van Slyke. The one added filtration and somewhat more complicated reagents of the latter are, perhaps, compensated by the increased sensitivity and permanence of the end-point.

Greenwald and Gross (8) determined blood chlorides, after removal of the proteins by a number of common procedures. These authors found that, of the procedures tried, only the Folin-Wu precipitation with tungstic acid and the open Carius nitric acid technique uniformly yielded solutions in which all the blood chloride could be determined. Other procedures for protein removal may not, therefore, be substituted for these, unless control analyses show that such substitution introduces no error.

CHLORIDE IN URINE. MODIFIED VOLHARD-HARVEY TITRATION (11)

The chloride is precipitated by means of silver nitrate in the presence of nitric acid, and the excess of silver is titrated with sulfocyanate. The titration may be carried out in the presence of the silver chloride precipitate with sufficient accuracy for the purposes of most urine analyses, although the end-point is an evanescent instead of a permanent color. In the reagents described, the ferric alum, silver nitrate, and nitric acid are combined together in one solution. This combination, used in the authors' laboratories, has an advantage over the original Harvey reagents, in which silver nitrate was in one solution and nitric acid with ferric alum in another. The combination makes one less reagent solution, and the HNO_3 stabilizes the $AgNO_3$.

Reagents

Standard silver nitrate solution. Dissolve 29.06 grams of silver nitrate in about 100 cc. of distilled water in 1-liter volumetric flask. Add 250 cc. of concentrated nitric acid and 250 cc. of a saturated aqueous solution of ferric ammonium sulfate. Dilute to a volume of 1 liter. One cubic centimeter of this solution is equivalent to 0.01 gram of sodium chloride.

Standard ammonium sulfocyanate solution. Dissolve about 6.5 grams of NH_4SCN in 800 cc. of distilled water. Titrate this solution against the above standard silver nitrate solution. Then calculate, as shown on page 30, the amount of water which must be added to the stock sulfocyanate solution to make it half the strength of the silver nitrate solution. One cubic centimeter of the silver nitrate solution should titrate 2 cc. of the ammonium sulfocyanate solution. One cubic centimeter of the ammonium sulfocyanate solution is equivalent to 0.005 gram of sodium chloride.

Procedure

To 5 cc. of urine, in a 250-cc. Erlenmeyer flask, add 100 cc. of water and 10 cc. of the silver nitrate solution. If the solution assumes a pinkish color, as it sometimes will, this may be dispelled by the addition of a few drops of saturated solution of potassium permanganate. Titrate with the sulfocyanate until the appearance of the first salmon pink or brownish tint that persists several seconds. If the urine contains an unusual concentration of chlorides this color may appear upon the addition of the first drop of sulfocyanate. In this case add another 10 cc. of the silver nitrate at once and resume the titration.

The most common cause of error (low results) is failure to recognize the *temporary nature of the end-point* (see p. 830). The *first color* that, for *a few seconds after one shaking,* permeates the solution, is the true end-point. When this is obtained the titration is finished.

In ketosis the urine may contain enough acetoacetic acid to give a red color with ferric iron, even before sulfocyanate has been added. The acetoacetic acid can be removed by acidifying the urine and boiling for a few minutes.

Subjects who have taken aspirin also excrete urines which form a red color with the ferric indicator (11a). In such cases an extra urine sample is employed as a control. The solution of standard silver nitrate and ferric indicator is added to both samples, and one of them is titrated with sulfocyanate until the red color becomes deeper than that of the control.

Calculation

$2A - B$ = grams of chloride calculated as NaCl per liter of urine.

$0.607\ (2A - B)$ = grams of Cl per liter of urine.

$17.1\ (2A - B)$ = milli-equivalents of Cl per liter of urine.

A = the cubic centimeters of silver nitrate added to the urine, and B the cubic centimeters of sulfocyanate used in the titration.

CHLORIDE IN FECES. OPEN CARIUS METHOD

Weigh 1 to 2 grams of the dried, pulverized stool, prepared by the method described on page 78, into a 250-cc. Pyrex glass Erlenmeyer flask. Add 10 cc. of the silver nitrate solution used in the determination of urine chlorides, and about 3 cc. of concentrated nitric acid. Cover the mouth of the flask with a watch glass or funnel and digest the mixture in a boiling water bath or steam bath for six hours, or until the solution becomes a clear yellow and the silver chloride has flocculated out completely. Allow the flask to cool, and titrate with sulfocyanate solution as in the urine chloride method.

If it is desired to determine both nitrogen and chloride in the feces, the latter may be preserved with sulfuric acid in the usual manner described on page 78. A suitable weighed aliquot of the stool paste may be subjected to the procedure just described for the analysis of dried stools. The presence of sulfuric acid does not interfere with the chloride determination.

The digestion of feces with nitric acid and silver nitrate can, like that of blood, be accelerated by boiling over a free flame with the addition of saturated potassium permanganate.

CHLORIDE IN 1-CC. SAMPLES OF BLOOD OR SERUM. OPEN CARIUS METHOD AS APPLIED BY VAN SLYKE AND SENDROY (24), WILSON AND BALL (28), AND EISENMAN (4)

This method may also be used for tissues, and for other fluids, such as urine, gastric juice, etc.

Reagents

In the Van Slyke and Sendroy technique silver nitrate and nitric acid are combined in a single solution. Wilson and Ball add the two reagents separately, for reasons discussed above.

1. *Combined reagent of Van Slyke and Sendroy: 0.05* N *$AgNO_3$ in concentrated HNO_3.* Dissolve 8.495 grams of fused silver nitrate in a minimum

amount of water and make the solution up to 1 liter with concentrated nitric acid (specific gravity 1.4). This solution will keep indefinitely.

2. *Separate reagents of Wilson and Ball.*

A. 0.15 N silver nitrate. Dissolve 25.485 grams of fused silver nitrate in water and dilute the solution to 1 liter. This solution must be carefully protected from light. Even under these conditions it deteriorates. It must, therefore, be standardized from time to time or frequently renewed. It may be standardized gravimetrically, or by titration against standard HCl or KCl solution.

B. Concentrated nitric acid (specific gravity 1.4).

Saturated solution of potassium permanganate.

0.02 N sulfocyanate. Dissolve 1.6 grams of sodium sulfocyanate, 1.5 grams of ammonium sulfocyanate, or 1.9 grams of potassium sulfocyanate in about 900 cc. of water. Filter the solution if it is not clear. With this solution titrate 3 cc. of the 0.05 N acid silver nitrate solution (or 1 cc. of the 0.15 N aqueous silver nitrate solution (*A*) plus 3 cc. of concentrated nitric acid. Perform the titration under the conditions described below for blood analyses, adding 6 cc. of 5 per cent ferric alum solution as indicator. From the titration results calculate how much to dilute the sulfocyanate (see p. 30) in order that 7.54 cc. shall be required to titrate 3 cc. of the 0.05 N silver nitrate in HNO_3, or 1 cc. of the 0.15 N silver nitrate. (The extra 0.04 cc. is the excess of sulfocyanate required to give the end-point.) The stock solution of sulfocyanate is diluted to the calculated volume, and its accuracy is checked by repeating the titration. The titration value of the sulfocyanate should be redetermined at least one a fortnight, as it may change.

Five per cent solution of ferric alum. (Powdered ferric alum may equally well be used.)

Procedure

Introduction of silver nitrate and nitric acid. To 1 cc. of blood or plasma in a 25 by 200-mm. Pyrex glass test tube, add slowly and ***with constant stirring*** 3 cc. of 0.05 N silver nitrate in concentrated nitric acid. Wilson and Ball, for reasons discussed above, prefer to add 1 cc. of 0.15 N silver nitrate first and, after this has been thoroughly mixed with the serum, to introduce 3 cc. of concentrated nitric acid.[1]

[1] Sunderman and Williams (22) find that in analyses of *dried* blood or tissue if the concentrated HNO_3-$AgNO_3$ solution of Van Slyke and Sendroy is added directly, low chloride values are obtained. Apparently the cause is that some of the chloride is occluded by fat, which floats up on the surface of the nitric acid during digestion and escapes disintegration. If the dried material is first soaked in water, or in aqueous silver nitrate solution, before digestion with HNO_3-$AgNO_3$ mixture, correct results are obtained.

***Digestion on a steam bath. Van Slyke and Sendroy** (24).* The tube is covered with a watch glass and the lower part is immersed in a steam bath until the solution above the AgCl precipitate is clear and light yellow in color. For this purpose one to two hours suffice for serum, while whole blood requires twelve hours. Permanganate may be added to diminish the color of the solution, as in the digestion over the free flame described below, and the addition is desirable in analyses of whole blood. The digests of plasma, without permanganate, are of such light color that no appreciable gain in the accuracy of the end-point is achieved by using permanganate.

***Alternative digestion over free flame. Eisenman** (4).* Two additional cubic centimeters of concentrated nitric acid are added. The acid-silver nitrate digestion mixture in the Pyrex tube is brought to a boil over the free flame. Saturated potassium permanganate is added to the boiling mixture a few drops at a time. Digestion is complete when the digestion mixture becomes clear and colorless or when the brown color persists for as much as thirty seconds after the addition of the last drop of permanganate. The sides of the tube are now washed down with distilled water, the solution is boiled again and permanganate again added a drop at a time. Usually only 4 or 5 drops are necessary at this stage. It is impossible to decolorize completely digests of whole blood or of certain sera. Permanganate accelerates digestion, and provides more nearly colorless solutions for subsequent titration. By the use of this digestion procedure the determination of chloride can be completed in about fifteen minutes. If excess permanganate is added, the solution can be decolorized with a few crystals of oxalic acid.

Titration. When digestion is complete the tubes are cooled to room temperature or lower; 6 cc. of 5 per cent ferric alum or 5 to 6 cc. of water and about 0.3 gram of powdered ferric alum are added; and the excess silver nitrate is titrated with 0.02 N sulfocyanate until the first appearance of a pink color that persists fifteen seconds. It is important before titration to cool the contents of the tubes thoroughly, because the end-point of the titration becomes increasingly sharp as the temperature diminishes.

Fiske and Sokhey (5), in order to make the end-point more permanent and sensitive, practically remove the AgCl from the field of reaction by centrifuging the mixed AgCl-AgSCN precipitates just before the end-point is reached, and then add the last drops of standard sulfocyanate to the supernatant solution. The writers have not in general found this precaution

necessary. The AgCl becomes well coagulated during the nitric acid digestion, and under the conditions of the titration, performed with ordinary rapidity, reaction of AgCl with the first drop of sulfocyanate is not rapid enough to make the true end-point difficult to distinguish.

Calculation

$20\ (7.54 - A)$ = milli-equivalents of Cl in 1 liter of blood.

$71\ (7.54 - A)$ = milligrams of Cl in 100 cc. of blood.

$117\ (7.54 - A)$ = chloride calculated as milligrams of NaCl in 100 cc. of blood.

A = cubic centimeters of 0.02 N sulfocyanate used in the titration of the unknown solution.

CHLORIDE IN 0.2 CC. OF BLOOD OR PLASMA. PATTERSON'S (17) MICRO MODIFICATION OF THE OPEN CARIUS METHOD

The procedure as presented, has been somewhat modified by Pauline M. Hald in the laboratory of one of the authors (J. P.).

Reagents

Silver nitrate reagent, 0.01333 N silver nitrate solution in nitric acid (3 cc. of this reagent are equivalent to 1 cc. of 0.04 N). Dissolve 2.265 grams of fused silver nitrate in a minimal amount of water and make it up to 1 liter with concentrated nitric acid (specific gravity 1.4). This solution keeps indefinitely if reasonably protected.

Concentrated nitric acid.

Saturated solution of potassium permanganate.

Acetone.

Powdered ferric alum.

0.02 N alcoholic solution of sodium sulfocyanate. Dissolve 1.6 grams of sodium sulfocyanate in about 900 cc. of 95 per cent[2] alcohol. With a microburette, graduated in 0.01 cc. divisions, titrate with the sulfocyanate 3 cc. of the silver nitrate reagent solution and 2 cc. of acetone, using about 0.3 gram of ferric alum as indicator. The sulfocyanate is then so diluted that 2 cc. are equivalent to 3 cc. of the silver nitrate solution. The sulfocyanate should be restandardized against the silver nitrate each day. The alcohol evaporates so readily that it is difficult to prevent the solution from becoming more concentrated on standing.

[2] Patterson recommends absolute alcohol, but 95 per cent seems to be quite satisfactory.

Procedure

Draw 0.2 cc. of blood or serum into a pipette calibrated ***to contain.*** Rinse the pipette into 1 cc. of distilled water in a 25 by 200-mm. Pyrex glass test tube. Then add slowly and with constant stirring 3 cc. of acid silver nitrate reagent and a few crystals of ferric alum. Heat the mixture to a gentle boil over a free flame. To the boiling solution add saturated potassium permanganate a drop at a time until the brown color produced by a drop of the solution persists for thirty seconds. Cool the solution to room temperature or below and add 2 cc. of acetone. Cool again. Titrate with 0.02 N alcoholic sulfocyanate from a micro burette. The end-point is neither the first appearance of brown color nor the maximum color obtained by excess sulfocyanate. It is necessary to practice the titration with known chloride solutions in order to learn the color change that must be used as end-point to give exact results.

Precautions must always be taken to prevent error from concentration of the alcoholic sulfocyanate solution, from which the alcohol evaporates rapidly on exposure to air.

Calculation

$100\ (2 - A)$ = milli-equivalents of Cl per liter of blood or serum.

$355\ (2 - A)$ = milligrams of Cl per 100 cc. of blood or serum.

$585\ (2 - A)$ = milligrams of Cl, as NaCl, per 100 cc. of blood or serum.

A = the cubic centimeters of 0.02 N sulfocyanate used in titration of the unknown solution.

CHLORIDE IN 0.02 CC. OF BLOOD OR PLASMA. CLAUDIUS' (2) ULTRA-MICRO APPLICATION OF THE OPEN CARIUS METHOD

Reagents

0.04 N silver nitrate. Dissolve 6.795 grams of fused silver nitrate in water and dilute to a liter.[3] This solution must be carefully protected from the light. Even under these conditions it deteriorates and must, therefore, be renewed or standardized at intervals.

Concentrated nitric acid (specific gravity 1.4).

Four per cent ferric nitrate solution acidified with nitric acid.

[3] It would probably be possible to combine silver nitrate and nitric acid into a single solution which would not deteriorate, as is done in the reagents of Van Slyke and Sendroy and of Patterson, described above.

Four per cent potassium permanganate solution.

0.005 N potassium sulfocyanate in absolute alcohol. Such a solution contains a little less than 0.5 gram of the salt per liter. It can be prepared by dissolving the salt in absolute alcohol in about these proportions and standardizing it against the silver nitrate by the titration procedure described below for the analysis of serum. It must be restandardized each day. 1.6 cc. should be equivalent to 0.2 cc. of the 0.04 N silver nitrate.

Absolute alcohol.

Procedure

0.02 cc. of blood is drawn up into a capillary pipette, calibrated ***to contain***, such as is used for hemoglobin determinations, and is transferred to a test tube of Pyrex or Jena glass containing a few drops of water. The pipette is washed out with a little water and the washings are added to the tube. 0.20 cc. of the 0.04 N silver nitrate solution are are similarly added, and 4 to 5 drops of pure concentrated nitric acid. The mixture is warmed over a small flame without boiling and with continual shaking of the tube. The protein coagulum dissolves quickly and only the silver nitrate and chloride remain. When the yellow fluid begins to clear it is heated to a boil with continued shaking of the tube, and when only about 0.25 cc. of fluid is left a drop of permanganate solution is added to decolorize the solution. If a little manganese dioxide is precipitated a drop of nitric acid is added and the solution is boiled a moment. After cooling, 5 cc. of absolute alcohol and a drop of 4 per cent ferric nitrate are added. The unprecipitated silver is now titrated with the 0.005 N sulfocyanate solution.

A micro burette divided into 0.01 cc. divisions is used, with a fine capillary glass tip which is vaselined in order to make the drops as small as possible. Such a tip delivers 150 to 200 drops of alcoholic solution per cubic centimeter. By means of a cock at the top which can be closed when the burette is not being used evaporation of alcohol can be prevented. Special mico-burettes of the types devised by Bang and by Shohl are adapted for such titrations (see chapter I).

In order to titrate to a standard color, after part of the sulfocyanate (e.g., 0.5 cc.) solution has been added, the titrated solution may be divided into two parts in two tubes of about 10 cc. capacity each. To one tube sulfocyanate is added until a perceptible permanent pink differentiates it from the other. Cyanate is then added to the remaining tube until it has the same pink color.

The end-point is very sharp. Presumably because of difficulty in measuring the small volumes of blood and silver nitrate with great accuracy, the method gives results deviating by ± 2 to 3 milli-equivalents from the mean. With larger samples, 0.1 cc. of blood, and 1.0 cc. of silver solution, the deviation from the mean was only ± 1 milli-equivalents. (Unpublished analyses of Dr. Alma Hiller.)

It is necessary to check the method by analysis of known 0.1 M chloride solutions: the results are likely to exceed the theoretical by a constant amount, perhaps because of amounts of chloride in the reagent too slight to be precipitated in blank. In case such a difference is found, results calculated as described below are to be corrected by subtracting the correction indicated by the check.

Calculation

$250\ (B - A)$ = milli-equivalents of Cl per liter of blood.

$1462\ (B - A)$ = chloride calculated as milligrams of NaCl per 100 cc.

$862\ (B - A)$ = milligrams of Cl per 100 cc.

B = cubic centimeters of sulfocyanate required in blank analysis to titrate the 0.2 cc. of 0.04 N silver nitrate. A = cubic centimeters of sulfocyanate used in blood analysis.

CHLORIDE IN FOLIN-WU BLOOD FILTRATES. WHITEHORN'S APPLICATION OF THE VOLHARD TITRATION (27)

Reagents

Solutions for Folin-Wu tungstic acid precipitation of proteins (see p. 65).

The *sodium tungstate solution* which is used for the precipitation of proteins should be tested for chloride in the following manner: Mix one volume of 10 per cent sodium tungstate solution with two volumes of concentrated chloride-free nitric acid, and filter the mixture into a test tube containing silver nitrate. If more than a slight cloud appears the tungstate is unsuitable for use. It may be freed from chloride by recrystallization from alcohol. A fifty per cent water solution of the contaminated tungstate is prepared with the aid of heat. It is cooled, and an equal volume of 95 per cent alcohol is slowly stirred in. After about ten minutes the crystallized tungstate is filtered on a Buchner funnel, washed twice with alcohol, and dried.

Concentrated nitric acid.

Powdered ferric alum.

0.03 N silver nitrate. Dissolve 5.097 grams of fused silver nitrate in water and make the volume up to 1 liter. (Instead of the 0.0282 N standard solutions in the original paper, 0.03 N solutions are here used, in order to facilitate calculation in milli-equivalents rather than grams of Cl.)

0.03 N sulfocyanate. Dissolve about 3.2 grams of potassium sulfocyanate, or 2.7 grams of sodium or 2.5 grams of ammonium sulfocyanate, in 1 liter of water. Mix 10 cc. of the tungstic acid mixture, 5 cc. of the silver nitrate solutions, 5 cc. of concentrated nitric acid, and 0.3 gram of ferric alum. Titrate the mixture with the sulfocyanate solution. Then adjust the strength of the latter by dilution so that 5.04 cc. are required to titrate 5 cc. of the silver solution. (0.04 cc. excess of sulfocyanate is required to give the end-point color.)

Procedure

Transfer 10 cc. of Folin-Wu tungstic acid filtrate of blood or serum, prepared in the usual manner (see p. 65), but with chloride-free tungstate, to a porcelain dish. To this add 5 cc. of silver nitrate solution and stir the mixture thoroughly. Add about 5 cc. of concentrated nitric acid, stir the mixture again and let it stand about 5 minutes. Then add with a spatula, about 0.3 gram of dry ferric alum and titrate the excess silver nitrate with sulfocyanate until the appearance of a pink color which persists for fifteen seconds.

Calculation

$$30\,(5.04 - A) = \text{milli-equivalents of Cl per liter of blood or serum.}$$

$$175.4\,(5.04 - A) = \text{chloride calculated as grams of NaCl per 100 cc. of blood or serum.}$$

A = the cubic centimeters of 0.03 N sulfocyanate used in the titration.

CHLORIDE IN FOLIN-WU BLOOD FILTRATE. IODOMETRIC TITRATION OF MCLEAN AND VAN SLYKE (14)

The method as here described differs from the original technique of McLean and Van Slyke (14) and Van Slyke and Donleavy (23) only in that it is applied to the Folin-Wu filtrate, instead of to the heat coagulation or picric acid filtrate, and in that the concentrations of the standard solutions are slightly altered in order to facilitate calculation of the results in terms of milli-equivalents of chloride instead of milligrams.

This titration has been applied to the Folin-Wu filtrate by Gettler (7), Hanna (9), and Short and Gellis (19). For discussion, see p. 832.

Reagents

Folin-Wu tungstate solution tested for chloride as described above for the Whitehorn method.

0.03 N silver nitrate in 25 per cent nitric acid solution. 5.097 grams of silver nitrate are weighed into 1-liter flask and dissolved in some water. Two hundred and fifty cubic centimeters of concentrated nitric acid (specific gravity 1.42) are added, and the solution is diluted to 1 liter with water.

In place of silver nitrate, metallic silver, which can be obtained in a high state of purity, may be used. In this case 3.236 grams of silver are dissolved in some of the concentrated nitric acid in a 250-cc. Erlenmeyer flask. Water is then added and the solution is boiled to expel nitric oxide fumes. The solution is then transferred to a 1 liter volumetric flask, and the rest of the 250 cc. of concentrated nitric acid are added, followed by water to bring the volume to 1 liter. If assurance of the greatest accuracy is desired, one will either use metallic silver for the standard solution, or at least prepare a small amount from metallic silver and with it check the main solution prepared from silver nitrate.

0.012 N potassium iodide solution. This solution contains 1.9924 grams of pure KI per liter. It is made up with 2.1 grams per liter, and is diluted to the extent indicated by a preliminary standardization against the 0.03 N silver nitrate. The standardization is carried out as follows:

Five cubic centimeters of the 0.03 N $AgNO_3$ solution, measured with a pipette accurate to 0.01 cc., are mixed with 5 cc. of water and 5 cc. of the starch citrate solution described below. Then, from a 25-cc. burette graduated in 0.05 cc. divisions, iodide is run in until a permanent blue end-point is obtained. The amount of exactly 0.012 N iodide that would be required is 12.65 cc., 12.50 cc. to precipitate the silver and 0.15 cc. to give the end-point (see below under "End-point"). Somewhat less of the approximately 0.013 N preliminary iodide solution will be required.

The extent to which the preliminary iodide solution must be diluted to make it exactly 0.012 N is calculated as follows: If A cc. of iodide are used in the titration, the preliminary solution is diluted to $\frac{12.50}{A - 0.15}$ times its volume; e.g.: If 11.55 cc. of iodide are used in the titration, and 950 cc. of preliminary solution are to be diluted to 0.012 N, the volume of the diluted solution will be $950 \times \frac{12.50}{11.40} = 1041$ cc. One will therefore add 91 cc. to

the preliminary iodide solution. The final iodide is checked by titrating again against the silver.

Buffered starch nitrite solution

Trisodium citrate, $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}\ H_2O$	446 grams
Soluble starch	2.5 grams
Sodium nitrite	19 grams
Water to	1000 cc.

This solution contains sodium citrate in 1.25 molecular concentration, and nitrite in 0.25 molecular. The total Na concentration is 4 equivalents per liter, 3.75 from the tri-sodium citrate and 0.25 from the nitrite. The citrate serves to neutralize the free nitric acid and bring the pH to the point best suited for development of blue color by reaction of the starch indicator with iodine. Four cubic centimeters of solution, containing 16 milli-equivalents of Na, serve to react with 1 cc. of concentrated (16 N) nitric acid, and to give a solution having the optimum acidity for the starch-iodine end-point.

To prepare the solution, the starch is first dissolved in water, to which the citrate and nitrite are then added, the solution being finally made up to 1 liter. In preparing the starch solution, the solution must be boiled. If soluble starch is used, a few minutes boiling suffice. If potato or corn starch is used, it should be boiled for at least an hour.

Proposed modifications of the reagents. The following modifications have been proposed. The writers can not at present offer from experience an opinion as to whether they are definite improvements on the original reagents for routine work.

Smith (21) includes in the citrate-nitrite-starch solution 80 cc. per liter of concentrated syrupy phosphoric acid, which, he finds, intensifies the blue of the end point.

Christy and Robson (1), in place of the citrate-nitrite-starch solution, use two solutions, viz., a 1 per cent solution of soluble starch and a 0.01 N solution of potassium bi-iodate. With this solution in place of nitrite, the citrate buffer becomes unnecessary. To the filtrate from the precipitated AgCl 1 cc. each of the potassium bi-iodate and of the starch solution is added, and the standard KI solution is run in from a burette until the usual blue end-point is reached.

Procedure

For duplicate titrations, 20 cc. of the Folin-Wu blood or plasma filtrate, representing 2 cc. of blood or plasma, are pipetted into a 50-cc. volumetric flask. Ten cubic centimeters of the 0.03 N silver nitrate solution are added, and the mixture is diluted to the 50-cc. mark. Two drops of caprylic alcohol are added to promote coagulation of the silver chloride, which is further assisted by inverting the stoppered flask

several times. After allowing five minutes for complete coagulation to occur, the solution is filtered through a dry folded filter. If the first drops of filtrate are cloudy they are passed through the filter a second time.

Of the filtrate two aliquot portions of 20 cc. each are pipetted into 50-cc. flasks. Four cubic centimeters of the starch-nitrite solution are added to each portion. A slight turbidity may appear at this point, but it does not interfere with the titration.

For the titration the 0.012 N KI solution is run in, from a burette graduated into 0.05 cc. divisions, until a ***permanent*** blue color appears.

Calculation

15 (10.15 − KI) = milli-equivalents of Cl per liter of blood or plasma.

87.7 (10.15 − KI) = chloride calculated as milligrams of NaCl per 100 cc.

The end-point (23)

Only a *permanent* and unmistakable blue is taken as the end-point. If the iodide is run in rapidly near the end of the titration, iodine may be formed more rapidly than the silver nitrate precipitates it, and a false end-point may be reached, which disappears after a few seconds' shaking. If, towards the end of the titration, the iodide is added in the usual manner, slowly, the blue shade caused by each drop disappears as soon as the solution is rotated, until the genuine end-point is reached. The latter is permanent, and in fact deepens with time.

The first appearance of the end-point is observed easily against a white background, but even more readily against one which matches the light yellow color of the silver iodide precipitate. The titration may be performed over a sheet of light yellow paper which lies on the table beneath the burette.

The fact that 0.15 cc. of excess KI solution is required to give the end-point does not detract from the accuracy of the titration, because, with a given volume of solution, the required excess is constant and sharply defined. The end-point is not approached gradually, but appears suddenly on the addition of the last drop of the 0.15 cc. excess of KI. This may be demonstrated in a control solution by mixing 1 cc. of HNO_3 of 1.42 specific gravity with 4 cc. of starch-citrate indicator, diluting up to 25 cc., and titrating with the KI solution. The first 0.10 cc. produces no color at all, but the next drop suddenly causes a definite blue which deepens rapidly for several seconds. The amount of excess KI required to produce the end-point

varies directly as the volume of the solution; consequently it is desirable to keep the volume at the end of the titration within approximately the same limits (25 to 30 cc.) in standardizing the sulfocyanate and in performing the analyses.

BIBLIOGRAPHY

1. CHRISTY, R. K., AND ROBSON, W.: The determination of chloride in biological fluids. *Biochem. J.*, 1928, **22**, 571.
2. CLAUDIUS, D.: Methode zur Mikrobestimmung des Chlors im Blut und anderen albuminhaltigen Medien. *Verhandl.* **34** *Kongresses Deutsch. Ges. inn. Med.*, Wiesbaden, 1922, p. 138.
3. DOUGHTY, H. W.: Mohr's method for the determination of silver and halogens in other than neutral solutions. *J. Am. Chem. Soc.*, 1924, **46**, 2707.
4. EISENMAN, A. J.: A note on the Van Slyke method for the determination of chlorides in blood and tissue. *J. Biol. Chem.*, 1929, **82**, 411.
5. FISKE, C. H., AND SOKHEY, S. S.: Ammonia and fixed base excretion after the administration of acid by various paths. *J. Biol. Chem.*, 1925, **63**, 309.
6. FOSTER, G. L.: A modification of the McLean-Van Slyke method for the determination of chlorides in blood. *J. Biol. Chem.*, 1917, **31**, 483.
7. GETTLER, A. O.: A method for the determination of death by drowning. *J. Am. Med. Ass.*, 1921, **77**, 1650.
8. GREENWALD, I., AND GROSS, J.: Some sources of error in the determination of chlorides in blood and similar material. *J. Biol. Chem.*, 1922, **54**, 589.
9. HANNA, M. I.: A modification of the McLean-Van Slyke method for the estimation of chlorides in blood. *J. Lab. and Clin. Med.*, 1927–28, **13**, 651.
10. HARDING, V. J., AND MASON, E. H.: The estimation of chlorides in body fluids. *J. Biol. Chem.*, 1917, **31**, 55.
11. HARVEY, S. C.: The quantitative determination of the chlorides in the urine. *Arch. Int. Med.*, 1910, **6**, 12.

11a. JEFFREY, W. H.: The Volhard-Harvey method for the estimation of chlorides in urine. *J. Lab. Clin. Med.*, 1927, **13**, 687.

12. KORÁNYI, A.: Physiologische und klinische Untersuchungen über den osmotischen Druck thierischer Flüssigkeiten. *Z. klin. Med*, 1897, **33**, 1.
13. LAUDAT, M.: Le dosage du chlore dans le serum sanguin et dans les liquides albumineux de l'organisme. *J. pharm. chim.*, 1917, **16**, 168.
14. McLEAN, F. C., AND VAN SLYKE, D. D.: A method for the titration of small amounts of halides. *J. Am. Chem. Soc.*, 1915, **37**, 1128. A method for the determination of chlorides in small amounts of body fluids. *J. Biol. Chem.*, 1915, **21**, 361.
15. MOHR, F.: Lehrbuch der Titrirmethode, 1856, II, 13.
16. MORACZEWSKI, W.: Die Mineralbestandtheile der menschlichen Organe. *Z. f. physiol. Chem.*, 1897, **23**, 483.
17. PATTERSON, J.: Determination of chloride in body fluids. *Biochem. J.*, 1928, **22**, 758.
18. ROTHMUND, V., AND BURGSTALLER, A.: Über die Genauigkeit der Chlorbestimmung nach Volhard. *Z. anorg. Chem.*, 1909, **63**, 330.
19. SHORT, J. J., AND GELLIS, A. D.: Blood chloride methods. *J. Biol. Chem.*, 1927, **73**, 219.

20. Smirk, F. H.: Micro-estimation of chlorine in whole blood, serum, or corpuscles. *Biochem. J.*, 1927, **21**, 31.

21. Smith, M.: The determination of chlorides in trichloroacetic acid filtrates from whole blood and plasma. *J. Biol. Chem.*, 1921, **45**, 437.

22. Sunderman, F. W., and Williams, P.: Diminution in chloride measurement after drying blood and tissues. *J. Biol. Chem.*, 1921, **92**, 99.

23. Van Slyke, D. D., and Donleavy, J. J.: A simplification of the McLean-Van Slyke method for determination of plasma chlorides. *J. Biol. Chem.*, 1919, **37**, 551.

24. Van Slyke, D. D., and Sendroy, J., Jr.: The determination of chlorides in blood and tissues. *J. Biol. Chem.*, 1923, **58**, 523.

25. Volhard, J.: Die Silbertitrirung mit Schwefelcyanammonium. *Z. anal. Chem.*, 1878, **17**, 482.

26. Wetmore, A. S.: Determination of chlorides in blood. *J. Biol. Chem.*, 1920, **45**, 113.

27. Whitehorn, J. C.: A system of blood analysis. Supplement II. Simplified method for the determination of chlorides in blood or plasma. *J. Biol. Chem.*, 1921, **45**, 449.

28. Wilson, D. W., and Ball, E. G.: A study of the estimation of chloride in blood and serum. *J. Biol. Chem.*, 1928, **79**, 221.

CHAPTER XXXI

Phosphorus

DISCUSSION

GRAVIMETRIC METHODS

Precipitation as magnesium ammonium phosphate, with weighing as $Mg_2P_2O_7$ or as $MgNH_4PO_4 \cdot 6H_2O$. The standard precipitate used for exact phosphate determinations is ammonium magnesium phosphate, which is formed as $MgNH_4PO_4 \cdot 6H_2O$. It must be precipitated under fairly exact conditions, or the proportions of Mg and NH_4 may vary from those in the formula. It is not adapted to micro analyses, but serves well for phosphate determinations in urine or feces. For weighing, the usual procedure is to ignite the precipitate at a white heat (about 1100°) and change it to the pyrophosphate, $Mg_2P_2O_7$. Jones (30) has found that the precipitate can be dried in air to constant weight, in about twenty hours at room temperature and more rapidly at 40°, and can then be weighed as $MgNH_4PO_4 \cdot 6H_2O$. This procedure has not been adopted by other analysts, but it seems that it could conveniently replace ignition when an electric muffle is not available for the latter.

Precipitation and weighing as ammonium phosphomolybdate. The characteristic yellow precipitate is obtained by warming or shaking phosphoric acid with an excess of ammonium molybdate in acid solution. The precipitate formed in the presence of nitric acid has the approximate composition $(MoO_3)_{12}PO_4(NH_4)_3(HNO_3)_2 \cdot H_2O$, with a molecular weight of 2019 (28). If formed in the presence of HCl or H_2SO_4 one of these acids replaces HNO_3 in the precipitate. The ammonia in the molecule is loosely bound. If the precipitate is washed with 1 per cent KNO_3 solution, as is done in some methods where the precipitate is to be measured by titration with alkali, nearly all the ammonia is removed in the washing (54). Even when the precipitate is formed under the constant conditions of Lorenz (36) and washed with alcohol, the NH_4 content is not exactly $(NH_4)_3$, is likely to be more nearly $(NH_4)_{3.5}$, with a variation of as much as ± 10 per cent (54). The other constituents of the precipitate also tend to vary, with so much fluctuation in the P content that use of the phosphomolybdate for exact gravimetric analysis was long considered impossible. The orthodox gravimetric procedure was merely to use the molybdate, when necessary, to separate phosphoric acid from substances which might interfere with the

precipitation as $MgNH_4PO_4 \cdot 6H_2O$. The molybdophosphate was then redissolved in ammonia, from which the magnesium salt was precipitated. In 1901, however, Lorenz (36) prescribed conditions for precipitating and drying molybdophosphate which yielded a precipitate of constant phosphorus content. Washed with alcohol and ether, or with acetone (42), and dried thirty minutes at room temperature *in vacuo* over sulfuric acid, the precipitate contains 3.295 per cent of P_2O_5, or 1.44 per cent of P. Lieb (35) finds that in micro determinations of 1 to 2 mg. of P, the P content of the precipitate averages 1.452 per cent. The accuracy of the method has been confirmed by Neubauer (42), Kuhn (32), and others. Kuhn (32), weighing the precipitate to within 0.01 mg. on a micro balance, obtained results with not over 1 per cent error from samples with only 0.03 mg. of P. The method has been adopted by Pregl (47) for micro-analyses. Our omission of it among the methods described in this chapter is due only to the reason that the *strychnine* molybdophosphate precipitate, next described, has been found even better adapted to micro gravimetric analyses of biological material.

Precipitation and weighing as strychnine phosphomolybdate. When phosphoric acid and molybdate are mixed in the presence of an alkaloid base instead of ammonia, a precipitate of alkaloid phosphomolybdate forms, in which the alkaloid replaces the ammonia in the molecule. Pouget and Chouchak (46) found strychnine to be an alkaloid particularly suitable for the precipitation. Embden defined conditions for its use in gravimetric analyses (20). For micro analyses strychnine phosphomolybdate has the following advantages over the ammonium phosphomolybdate:

1. The weight of the strychnine precipitate is greater. Per 1 mg. of P the ammonium phosphomolybdate weighs 69 mg. and the strychnine phosphomolybdate 89.3 mg.

2. The strychnine precipitate is more insoluble and forms much more rapidly.

3. The strychnine precipitate readily coagulates into large particles which can be more easily handled than the ammonium precipitate, and have less tendency to adhere to the walls of the precipitating vessel.

4. The strychnine precipitate can be dried to constant weight in an ordinary drying oven at 100° or 110°.

Fetter (21) and Myrbaeck (41) have applied the strychnine molybdate precipitation to gravimetric determination of the inorganic phosphate in the trichloroacetic acid filtrates of blood and plasma, and consider it the most accurate of micro methods for these analyses. Filtrate equivalent to 2.5 cc. of normal plasma gives 8 to 10 mg. of precipitate.

TITRATION METHODS

Of the titration methods only one, that with uranium, is performed without previous isolation of the phosphate. In the other methods the phosphoric acid is first precipitated in one of the three ways outlined above, and the precipitate is measured by titrating the amount of acid the $MgNH_4PO_4$ neutralizes, or the amount of alkali the molybdate precipitates neutralize, or the amount of permanganate the molybdenum will reduce, after the Mo^{vi} has been first reduced to Mo^{iii} by the action of metallic Zn or Al.

The uranium titration

This procedure, described in 1859 by Pincus (45), has been applied, with slight modifications, to analysis of urine, fertilizer, and other materials. It is the simplest and most rapid of phosphate determinations. Albumin and sugar do not interfere. It depends upon the fact that when a uranium salt is added to a hot phosphate solution at pH not below 5, the uranium is instantly precipitated, the uranium and phosphoric acid entering the precipitate in the proportions indicated by the formula, $Ur_2O_3(PO_4)_2$. Uranium acetate or nitrate solution is added until an excess of uranium can be detected in the titrated solution. Pincus detected this end point by mixing successive drops of the titrated solution on white porcelain with ferrocyanide, which forms a brown color as soon as unprecipitated uranium is present. Malot (37), in order to obviate this somewhat tedious procedure, added cochineal to the titrated solution. Cochineal forms a green compound with uranium as soon as excess of the latter has been added. Consequently the color changes from the red of cochineal in acid solution to the green of the uranium-cochineal compound. The end-point is more sensitive than that of ferrocyanide to excess uranium. One or 2 drops of uranium solution added to 75 cc. of water suffice to cause the complete color change of cochineal, but 0.3 to 0.4 cc. are required to make a drop of the water give a marked color with ferrocyanide.

With urine some analysts prefer a combination of the two indicators. Cochineal is used in the titrated solution to indicate, by the green color formed, the approach of the end-point. Then tests with ferrocyanide are begun after addition of each 0.1 cc. of uranium solution. The chief essential is that the standardization of the uranium solution and the determination of the blank correction, for the amount of excess uranium to give the end point, shall both be carried out with the same end-point and under the same conditions used in subsequent analyses.

For the precipitation of uranyl phosphate a reaction of about pH 5 is

required. This is obtained by adding an acetate buffer solution to the urine. If the method is applied to solutions containing free mineral acid (e.g., the H_2SO_4 in the ash solution obtained by wet combustion of feces, or the HCl in extract of Stolte ash), the acid should be neutralized before the buffer acetate is added.

Du Parc and Rogovine (19) recommend sodium salicylate as indicator, the titrated mixture being 10 cc. of urine, 40 cc. of water and 10 cc. of 10 per cent sodium salicylate solution, while the uranium is used 5 times as dilute as usual. The writers have found the end-point usable in colorless phosphate solutions, but not with strongly pigmented urines, despite the dilution designed to prevent interference of the urinary pigments.

Acidimetric titration of precipitated ammonium magnesium phosphate

At pH 4.5 to 4.8 practically all of the phosphate in dilute solutions is in the form BH_2PO_4. Several authors, already quoted in the magnesium chapter, have utilized this fact to titrate the precipitate. The reaction can be indicated by the equation:

$$MgNH_4PO_4 + 2\ HCl = MgCl_2 + (NH_4)H_2PO_4$$

Each molecule of phosphate neutralizes, at the end point of pH 4.5 to 4.8, two equivalents of acid. The titration is carried out by dissolving the precipitate in excess of standard HCl and titrating back with standard NaOH. The titration can be used with as little as 2 mg. of phosphorus. Because of the preliminary handling of the precipitate, the analysis requires much more time than the uranium titration, but can be used when sufficient material is not available for the latter, which requires at least 10 mg. of phosphorus to give accurate results.

Alkalimetric titration of the ammonium phosphomolybdate precipitate

From the approximate formula of the precipitate (28), which may be written $(NH_4)_3PO_4 \cdot 12H_2MoO_4 \cdot 2HNO_3$, it is evident that the molecule contains 26 acid hydrogen atoms. The precipitate can accordingly be titrated with standard alkali. Indicators, like phenophthalein or thymol blue, which change color at pH a little above 8, are used, in order to obtain complete neutralization of the H_2MoO_4. At this pH, however, ammonia acts as a buffer, and diminishes the sharpness of the end-point. Consequently in the most exact applications of this titration (32, 43, 51) the precipitate is dissolved in excess of 0.1 N NaOH and the ammonia is boiled off before the final titration, which is carried out by adding 0.1 N HCl, and

again 0.1 N NaOH to the end point. Under these conditions the titration reaction is represented by the equation:

$$H_3PO_4 \cdot 12\ H_2MoO_4 \cdot 2\ HNO_3 + 28\ NaOH = Na_2HPO_4 + 12\ Na_2MoO_4 + 2\ NaNO_3$$

According to this equation 1 molecule of phosphoric acid is titrated by 28 equivalents of alkali, and 1 cc. of 0.1 N NaOH titrates $\frac{3.1}{28} = 0.111$ mg. of phosphorus. Because the acidity of the phosphoric acid is multiplied 14-fold by the H_2MoO_4 and HNO_3 attached to it, this titration is adaptable to micro as well as macro analyses, and has been used with samples containing as little as 0.1 mg. of P (32, 43).

Instead of removing the ammonia by boiling, Bang (3) combined it with formaldehyde by adding the latter in 40 per cent solution before the titration was completed. Hammarsten (26) obtained a similar effect, with sharper end-point, by adding 2 volumes of 99 per cent acetone, in the presence of which ammonia becomes inert to the thymolphthalein used as indicator.

Because carbonic acid neutralizes at the end-point of the reaction an entire equivalent of alkali to form $NaHCO_3$, it is important, especially in micro analyses, to use CO_2-free standard alkali and work under conditions preventing interference of atmospheric CO_2 with the end-point. Kuhn (32), after boiling off the ammonia with excess 0.1 N NaOH, adds excess 0.1 N acid and boils again, in order to remove CO_2, before the final titration.

In the preliminary handling of the precipitate, details of precipitation and washing must be followed minutely or variation in the composition of the precipitate will cause variation in the results. In Pemberton's (44) macro form of the method, which is recognized by the American Association of Agricultural Chemists, (1), the precipitate is washed with 1 per cent KNO_3 solution, and is then redissolved in alkali and titrated without boiling off the ammonia. Under the conditions employed, 1 mole of PO_4 titrates 23 (instead of 28) moles of NaOH, so that 1 cc. of 0.1 N alkali is equivalent to $3.1/23 = 0.135$ mg. of P instead of the 0.111 mg. found under the conditions used by European analysts. In Shohl and Brown's form of the method, given for stools in this chapter, 1cc. of 0.1 N alkali is equivalent to 0.119 mg. of P. This difference in factors indicates the empirical nature of the titration.

The titration, as developed by Neumann (43) for analyses in combination with wet digestion of urine, feces, and other material (see p. 69) and by Pemberton (44) for agricultural analyses (1) has had a wide application. The data in the literature (26, 32, 43, 51, 52) leave no doubt that it can give

accurate results, both in macro analyses, and in micro analyses of amounts of phosphorus down to 0.1 mg. The empirical dependence of the composition and alkali neutralizing power of the precipitate on the conditions of precipitation makes it essential, however, to observe strictly the prescribed conditions in obtaining the precipitate, and the disturbing effect of CO_2 on the end-point must be guarded against, in order to avoid errors. The labor required equals that for gravimetric analyses with porous crucibles when modern equipment is available for ignition of $Mg_2P_2O_7$ or drying of the strychnine molybdate, and the sources of error are more numerous in this titration than in either of the gravimetric methods or the uranium titration. The ammonium phosphomolybdate titration requires less material than the uranium titration, and may be used for stools when the available amount is limited, and a volumetric method is required. For this purpose Shohl and Brown's application of the method will be described.

Alkalimetric titration of the strychnine phosphomolybdate precipitate

The strychnine molybdate precipitate can also be dissolved in excess of standard alkali and determined by titration. Myrbaeck (41) has utilized this technique. The alkaloid can not be removed, like ammonia, by distillation, and the titration must be carried out in its presence. Otherwise it is essentially the same as titration of the ammonium phosphomolybdate precipitate. Myrbaeck found that 1 millimole (31 mg.) of phosphorus was equivalent to 19 millimoles of NaOH in the titration, or that 1 cc. of 0.1 N NaOH was equivalent to $3.1/19 = 0.163$ mg. of P. Because of the convenience with which the preliminary handling of the strychnine phosphomolybdate precipitate can be carried through, this titration promised an alternative to colorimetric methods for micro analyses of blood. The writers, however, have not been able to obtain consistent results with it.

Permanganate titration of molybdenum in phosphomolybdate precipitate

Denigés (13) has shown that Mo^{vi} can be reduced to MO^{iii} by boiling with aluminum foil in sulfuric acid solution, and that the reduced Mo^{iii} can be titrated back to Mo^{vi} by permanganate. Javillier and Djelatides (29) showed that this method can be applied to the redissolved ammonium phosphomolybdate precipitate. In this application, 1 cc. of 0.1 N permanganate is equivalent to 0.09 mg. of phosphorus. Javillier and Djelatides found that in determination of amounts of phosphorus exceeding 0.5 mg. the error was usually less than 1 per cent, but that if the sample contained only 0.1 mg. of P the error might be 5 or 10 per cent. They attributed the error to the difficulty of quantitative handling of small amounts of ammo-

nium phosphomolybdate precipitate rather than to the titration. The writers have not had experience with this method. Like the alkalimetric titration of the same precipitate, it appears to require minute attention to detail, and about the same number of operations.

COLORIMETRIC METHODS

For quantities of phosphorus less than 0.1 mg., such as are encountered in determining the PO_4 in 1 cc. samples of serum, the most practicable methods at present available without use of the microbalance are colorimetric. They are not so precise as the gravimetric methods carried out with larger amounts of material, and consequently colorimetric methods are recommended for urine and feces only when unusually small amounts are available.

With the Tisdall method (53), which in the authors' laboratories has given the best colorimetric results, the error can be kept within ± 2 or 3 per cent, and colorimetric comparisons can be carried out with accuracy when the unknown varies from 60 to 150 per cent as concentrated as the standard. With the modifications of the Bell-Doisy procedure the unknown should be within 20 per cent of the standard in order to avoid increasing the error, which is difficult to keep below ± 5 per cent.

All the methods that at present are in general use depend upon the measurement of the blue or green color which is developed when the Mo^{vi} of the molybdic acid is reduced to Mo^{iii} or intermediate forms.[1]

Colorimetric determinations on phosphomolybdate precipitates (*Tisdall*)

The reduction was at first applied to the molybdic acid precipitated as ammonium phosphomolybdate. Taylor and Miller (52) redissolved the precipitate, and reduced the Mo^{vi} by means of phenyl hydrazine. Tisdall (53) precipitated as strychnine phosphomolybdate, redissolved this in alkali, and reduced the Mo^{vi} with ferrocyanide, obtaining a greenish solution well adapted to colorimetry. The color is presumably a mixture of that of the blue molybdenum compound and the yellow of the ferricyanide produced

[1] The first colorimetric micro method appears to have been that of Pouget and Chouchak (46), who in 1905 precipitated the highly insoluble strychnine phosphomolybdate in very dilute solution, and measured the amount by the depth of the yellow color of the suspension while the latter was still colloidal. Greenwald (25) found the method satisfactory. Kober and Egerer (31) measured the same suspensions with a nephelometer, and their technique was adopted by Bloor (6, 7) for his studies of phospholipoids. These procedures appear at present to have been entirely displaced from general use by methods in which the blue color of reduced molybdenum is measured.

by the reaction from ferrocyanide. Since proportional amounts of the two colored products are produced, the resulting color is constant. Tisdall's method, published in 1922, has stood well the test of time. The preliminary precipitation and washing by centrifugation of the strychnine phosphomolybdate make the procedure longer than the direct determinations next discussed, but in the experience of the writers the relatively greater accuracy, and freedom from disturbing effects of other substances, make the Tisdall method the one of choice among the colorimetric procedures at present available. Convenience and accuracy in this method are augmented by the fact that the volume of the centrifuged strychnine phosphomolybdate serves as a close approximate measure of the amount of phosphate, and shows how much one must dilute the subsequent colored solution in order to match the standard most closely.

Direct colorimetry without precipitation (Bell-Doisy)

A more rapid technique is based on the fact, discovered independently by Bell and Doisy (4) and Denigés (13), that certain reducing agents will reduce the Mo^{vi} of phosphomolybdic acid, and at the same time have only negligible effect on the Mo^{vi} of uncombined molybdic acid in the same solution. As reducing agent, Bell and Doisy used hydroquinone with sulfite, while Denigés used stannous chloride. With these reagents it was not necessary to precipitate the phosphomolybdate. It sufficed to add molybdic acid and the reducing agent to the blood filtrate or other solution containing a few hundredths of a milligram of phosphate phosphorus, and the color developed at once. It was found that numerous substances if present in sufficient concentration could interfere with the color development (17, 50), but none appear to occur in disturbing amounts in blood filtrates. Bell and Doisy added sodium carbonate after the color had developed, apparently in order to prevent turbidity which occasionally resulted from traces of protein in the blood filtrate. Briggs (10) showed that if the color was developed and read in acid solution there was much less rapid fading, and that no turbidity formed if the blood had been shaken thoroughly with trichloroacetic acid to precipitate the proteins. Briggs' modification also obviated a bleaching effect, which was caused in the original Bell-Doisy procedure by oxalate and citrate in the amounts used as anti-coagulants (40, 15). In Denigés' method, as in the Briggs form of the Bell-Doisy, the color is developed and read in acid solution. Benedict and Theis (6) added another definite improvement, for the minute amounts of phosphate in blood, by heating the mixture during development of the color, thereby greatly increasing its intensity. Their method is applicable to filtrates

from plasma and serum, but cannot be used with whole blood filtrates, because the heat would cause hydrolysis of some of the organic phosphates present. The heating offers no advantage over the Briggs method for urine and feces, where plenty of phosphate is present.

Fiske and Subbarow (23) have used, in place of hydroquinone as reducing agent, 1, 2, 4-aminonaphthosulfonic acid, Leiboff (34) has used monomethyl-p-aminophenol, and Kuttner (33) stannous chloride. In the color produced, Fiske and Subbarow's reagent was found by them to be less affected than hydroquinone by various salts and by silicic acid, which with molybdate and hydroquinone gives nearly as much color as does phosphoric acid. Leiboff recommends his reagent for its stability. Atkins and Wilson (2) prefer Denigès' method, (14) with stannous chloride as reducing agent. For blood analyses, however, none of these reagents has yet gone through the test of general usage that has been met by hydroquinone. We therefore describe the Benedict-Theis modification of the Bell-Doisy method for general use in rapid micro-determination of inorganic phosphate, with the Briggs modification for use when heating must be avoided.

Roe, Irish and Boyd (50) have reviewed the *general precautions for the direct colorimetric molybdate method*, and summarized them as follows:

Reduction of the part of molybdic acid present which is not combined with phosphoric occurs to some extent: it is merely so much slower than reduction of phosphomolybdic acid that it can be made relatively negligible by properly fixing conditions. Unnecessary excess of uncombined molybdic acid, and also of reducing agent, are accordingly to be avoided in order to keep reduction of the free molybdic acid down to a negligible rate.

The intensity of color developed from phosphomolybdate depends upon the acidity and temperature of the solution. When developed by reduction of phosphomolybdate in hot solution (Benedict-Theis method), the color increases with increase of sulfuric acid from 0.1 N to 0.4 N, where it reaches a maximum. Further increase diminishes the color until at 0.9 N it is about 70 per cent as intense as it is at 0.4 N. A level plateau is then reached from 0.9 N to 1.4 N H_2SO_4, over which range the color is constant. Further increase rapidly diminishes the color. It is desirable to fix the sulfuric acid concentration near the middle of the plateau, at about 1.15 N H_2SO_4, in order to minimize the possibility of error from variations in acidity (50).

When the color is developed in the cold, however, as in Briggs' method, the optimum acidity for a stable color is about 0.5 N sulfuric acid (37).

When sulfuric and nitric acid are used to destroy organic matter preliminary to estimation of total phosphorus, the nitric acid must be completely removed before the colorimetric estimation. However, when the nitric

acid is driven off by boiling, heating of the residual sulfuric acid must not be carried too far, or phosphoric acid may be volatilized, or converted into metaphosphoric acid (55). The necessity for overheating to drive off nitric acid may be avoided by reducing the excess nitric acid with sulfite or by finishing the oxidation with hydrogen peroxide instead of nitric acid.

CHOICE OF METHODS

The following methods will be described:

	MILLIGRAMS OF P TO WHICH METHOD IS APPLICABLE	MATERIAL TO WHICH APPLICATION OF METHOD IS DESCRIBED
Weighing $Mg_2P_2O_7$	10 to 100	Urine and feces
Weighing strychnine molybdate. Embden	0.1 to 4	Blood, urine, and feces
Uranium titration. Pincus-Malot	10 to 50	Urine and feces
Acidimetric titration of $MgNH_4PO_4$. Fiske	2 to 7	Urine
Alkalimetric titration of ammonium phosphomolybdate. Shohl and Brown	5 to 20	Feces
Colorimetric on strychnine molybdate precipitate. Tisdall	0.02 to 0.1	Blood, urine, and feces
Colorimetric, direct. Benedict and Theis heating modification of Bell-Doisy	0.02 to 0.1	Blood, all P except cell inorganic
Colorimetric, direct. Briggs-Bell-Doisy	0.02 to 0.1	Whole blood or cell inorganic P

For *urine*, when plenty of material is available, as in analysis of twenty-four-hour specimens from adults, one may use either the gravimetric $Mg_2P_2O_7$ method or the uranium titration, each of which requires a 50-cc. sample. The gravimetric method is the one of choice when maximum accuracy is desired, and with modern Gooch or porous glass crucibles and an electric muffle it is a simple matter to do many analyses in a day. The uranium titration is somewhat less exact, the error being about 1 per cent when 50 mg. of P are in the sample, and greater in proportion as the phosphate content is less. This titration is, however, within its limits, serviceable and reliable, and is the simplest and most rapid of all phosphate determinations. Its accuracy is sufficient for most purposes, and it has justified its place as the most used method for clinical studies, which it has held consistently since its publication in 1859.

When it is necessary to analyze small samples of urine, Embden's method

provides an accurate gravimetric procedure, in which 5 cc. suffice for duplicate analyses. Somewhat less exact, perhaps, and requiring more manipulations, Fiske's method provides nevertheless a titration of elegance, requiring 2 to 10 cc. of urine. The smallest quantities can be analyzed by Tisdall's colorimetric method, by which duplicates can be performed on a 0.5 cc. sample if necessary, and with an error seldom exceeding ± 2 per cent.

For *feces*, after they have been ashed, the choice is in the same order as for urine. The ammonium molybdate titration of Shohl and Brown provides an additional titration for samples intermediate in size between those desirable for the uranium titration and for the micro methods.

For *blood and serum* the writers prefer either the Embden-Fetter gravimetric or the Tisdall colorimetric method, depending upon the amount of material available and the relative facilities for weighing or colorimetry in the laboratory. More rapid, but less exact, are the Benedict-Theis and Briggs direct colorimetric methods performed without preliminary precipitation of the phosphate. Of these, the Benedict-Theis is preferable, because of the greater intensity of the color which it offers with the small amounts of phosphorus present. For the inorganic phosphate in filtrates from whole blood, however, the Benedict-Theis method can not be used, because the heating would hydrolyze the acid-soluble organic phosphate present. For this fraction the Briggs method, or one of its modifications (23, 34) in which no heat is applied, can be employed for rapid approximate results.

Phosphorus in Urine

The greater part of the phosphorus in urine occurs as inorganic salts of alkali metals. A small and variable fraction, usually about 5 per cent, is in combination with organic substances, glycerophosphoric acid, phosphocarnic acid, etc. As a preliminary to determination of total phosphorus it is necessary to oxidize or hydrolyze the organic fraction. The inorganic phosphate, on the other hand, can be precipitated directly from the untreated urine.

Inorganic Phosphate of Urine, Gravimetric. Mackay and Butler's Modification of Mathison's (38) Method

The following procedure was developed in the Hospital of The Rockefeller Institute by Eaton M. Mackay and Allan M. Butler. It is a modification of a method described by Mathison (38) in which inorganic phosphorus is precipitated from the urine as magnesium ammonium phosphate, which

is ignited in the usual manner and weighed as the pyrophosphate. An excess of citrate prevents ionization of the calcium and minimizes contamination of the precipitate with calcium phosphate (16). The magnesium citrate mixture of Fiske (22) is utilized. The method can be applied even to albuminous urines.

Reagents

Concentrated hydrochloric acid.

Magnesium citrate mixture of Fiske. To 265 grams of citric acid dissolved in 350 cc. of hot water add with stirring 13 grams of magnesium oxide free from carbonate. Cool the solution and add 330 cc. of strong ammonia water (specific gravity 0.90). Cool and dilute to 1 liter.

Concentrated ammonia water (specific gravite 0.90).

Dilute ammonia solution. One volume of strong ammonia water diluted to 15 volumes with water.

Procedure

Sixty cubic centimeters or more of urine are acidified with a few drops of concentrated hydrochloric acid, to dissolve any precipitated phosphate. The acidified urine is filtered. To 50 cc. of the filtrate, in a 250-cc. Erlenmeyer flask of Pyrex glass, are added 10 cc. of the magnesium citrate mixture and 20 cc. of strong ammonia water. The flask, closed with a rubber stopper, is shaken vigorously for one to two minutes. It is then allowed to stand at least two hours, preferably over night. At the end of this time the crystalline precipitate is transferred to a weighed 15-cc. Jena glass crucible with a porous glass filtering disc of coarse porosity. The precipitate is washed with 100 cc. or more of dilute ammonia solution, which is also used, with the aid of a rubber-tipped stirring rod, to remove the portions of the precipitate which adhere to the flask.

After drying in an oven at 100° to 120° for one-half hour, the crucible is set in a large covered porcelain crucible and heated, either over a strong burner or in a muffle furnace, to a bright red heat (about 1100°).

A muffle is convenient when several analyses are done at a time. The crucibles are cooled, first to 150° in the muffle, finally in a desiccator, and weighed. After the intensity of heat necessary to ensure complete conversion to pyro phosphate has been fixed by experience, repeated heating to attain constant weight is unnecessary.

Calculation

$$0.2784\ W = \text{grams of P in sample.}$$

$$\frac{8978\ W}{V} = \text{millimoles of phosphate per liter of urine.}$$

$$\frac{278.4\ W}{V} = \text{grams of P per liter of urine.}$$

W = grams of $Mg_2P_2O_7$, V = cubic centimeters of urine (usually 50) in sample.

The possibility of avoiding ignition by weighing the air-dried precipitate as $MgHN_4PO_4 \cdot 6H_2O$ has been discussed in the introduction of this chapter.

INORGANIC PHOSPHATE IN URINE. GRAVIMETRIC METHOD OF EMBDEN (20)

For discussion of the principle of this method, see page 849. This method provides gravimetric accuracy when amounts of urine too small for the magnesium phosphate method are available.

Reagents

The same as for the determination of inorganic phosphate in blood by the Embden-Fetter method (p. 873).

Procedure

Five cubic centimeters of urine, well mixed to suspend any insoluble phosphates, are measured into a 50 cc. flask. Twenty cubic centimeters of water are added and the flask is filled up to mark with 10 per cent trichloroacetic acid. The acid serves to dissolve any precipitated phosphate and to precipitate any protein present.

If the solution is not clear it is filtered after ten minutes. Twenty cubic centimeters of filtrate are diluted to 60 cc. with water, and the rest of the determination is carried out as described on page 873 for inorganic phosphate in blood. A rare urine may contain more than 2 mg. of phosphorus per cubic centimeter. In such a case only 10 cc. of filtrate are used, as the reagents are adapted to precipitation of not more than 4 mg. of phosphate phosphorus.

Calculation

$$0.0112 \times \text{mg. precipitate} = \text{mg. P in sample.}$$

The amount of phosphorus found in the sample is multiplied by 500 if 20 cc. of filtrate were used, by 1000 if only 10 were taken, to calculate the phosphorus per liter of urine.

INORGANIC PHOSPHATE OF URINE. TITRATION WITH URANIUM ACETATE. PINCUS (45) AND MALOT (37)

Reagents

Uranium acetate standard solution, of which 1 cc. is equivalent to 2 mg. of phosphorus. Dissolve about 32 grams of uranyl acetate and 5 cc. of glacial acetic acid in water without the aid of heat and dilute to 1 liter. Titrate this solution against 50 cc. of a standard phosphate solution containing 4.388 grams of KH_2PO_4 per liter. This solution contains 1 mg. of P per cubic centimeter. In the titration use the technique described below for titration of urine, including correction for the blank. Adjust the uranium solution so that 25 cc. titrate 50 cc. of the phosphate solution.

Standard phosphate solution containing 1 mg. of P per cubic centimeter. Purest KH_2PO_4 is pulverized and dried for several days over sulfuric acid until its weight becomes constant. 4.388 grams are dissolved and made up to 1 liter in water. Five cubic centimeters of chloroform may be added to prevent mold formation. This standard solution is used also to prepare the more dilute standards used for colorimetric methods.

Acetate buffer solution. Dissolve 100 grams of sodium acetate and 30 cc. of glacial acetic acid in water and dilute to a liter.

A *10 per cent solution of potassium ferrocyanide.*

A *saturated solution of cochineal in 30 per cent alcohol.*

Procedure

To 50 cc. of urine in a 250-cc. Erlenmeyer flask add 5 cc. of the acetate buffer solution, and 1 cc. of cochineal solution. Heat to boiling. Remove the flask from the flame and run in uranium solution from a burette until the change from red through pinkish gray to definite green is complete in the titrated solution. To match the color of the end-point one may use another flask in which a similar titration has just been completed, and to which a definite excess of uranium solution has been added. If one is in doubt concerning the reaching of the end-point, it is well to reheat the flask. Either the green may become more definite, or the pinkish tint may become evident, indicating that more uranium must be added. If more than 25 cc. of uranium solution are needed, or the titration is delayed so that the solution cools below 60°, it should be reheated before the titration is finished. Precipitation of phosphate by uranium is not instantaneous unless the solution is hot.[2]

[2] In the original method applied to urine and other solutions containing calcium salts as well as phosphate, it was customary to add the greater part of the uranium to the solu-

To obtain an end-point of maximum sharpness it is necessary to have enough cochineal present so that its color will dominate that of the gray uranium phosphate suspension. The writers have found 1 cc. of the extract satisfactory, but the amount may vary with different extracts. It is advisable to prepare enough extract for a long supply, and decide by test the optimum amount to use. The end-point is somewhat easier to detect in titration of colorless solutions than in urine.

Some analysts prefer to use the ferrocyanide end-point. For this purpose it is most rapid and convenient to use cochineal to indicate the approach of the end-point, which is shown by cochineal before it is by the ferrocyanide reaction. One titrates until, from the color change of the cochineal in the solution, it is apparent that one is not far from the end. Then uranium solution is added, 2 or 3 drops at a time, After each addition the flask is rotated for several seconds, and then a drop of the titrated mixture is removed on a rod and mixed with a drop of ferrocyanide on a white plate. If the mixture assumes a darker color than a a control drop of ferrocyanide near it on the plate, the end-point has been reached. It is desirable to pass a vaselined cloth over the surface of the plate before the ferrocyanide test drops are placed. On a greased or waxed surface the drops retain a hemispherical shape, and show the color change better than if the fluid spreads out in a thin layer. A flat plate or tile serves as well as the special indented plates sold for the purpose.

If the urine is deeply bile-stained, it may, before the titration, be acidified with hydrochloric acid and decolorized by heating with a few drops of permanganate solution. The hydrochloric acid is then neutralized by adding a measured equivalent of ammonia or sodium hydroxide solution, the acetate buffer is added, and the titration is carried out.

The end-point is not quite so exact in urine as in unpigmented solutions. Even in urine, however, it appears that the titration error can be kept within 0.25 cc. of uranium solution, corresponding to 0.5 mg. of P, or 1 per cent of the amount determined when the urine content is 1 gram per liter. If the phosphate concentration is less, the per cent error is greater.

---

tion without heating the latter, because heat was likely to cause precipitation of calcium phosphate. The solution was heated only when the end-point was approached and the greater part of the phosphate had been precipitated by uranium. With the use of acetate buffer, however, setting the pH in the neighborhood of 5, there appears to be no danger of error from heating the solution before the titration is begun. In the hot solution the color changes indicating the approach of the end-point are more easily seen.

A blank is run, with 50 cc. of water in place of the urine, in order to ascertain how much uranium solution is required to give the end-point by whichever of the two methods is used to detect it.

If, for any reason, less than 50 cc. of urine are taken for an analysis, the volume is brought up to 50 cc. with water before the acetate solution is added.

Calculation

$2\ (A - B)$ = milligrams of phosphate phosphorus in sample.

$$\frac{2\ (A - B)}{V} = \text{grams of phosphate P per liter of urine.}$$

$$\frac{64.5\ (A - B)}{V} = \text{millimoles of phosphate per liter of urine.}$$

A = cubic centimeters of uranium solution to titrate sample, B = cubic centimeters to titrate blank, and V = cubic centimeters of urine in the sample.

INORGANIC PHOSPHATE OF URINE. TITRATION METHOD OF FISKE (22)

The principle has been discussed under "Acidimetric titration of precipitated ammonium magnesium phosphate."

Reagents

Magnesium citrate mixture. Dissolve 80 grams of citric acid in 100 cc. of hot water. Add 4 grams of magnesium oxide and stir the mixture until this is dissolved. Cool the solution, add 100 cc. of concentrated ammonia solution, dilute to 300 cc., set it aside for twenty-four hours, and then filter. (If the magnesium oxide contains much carbonate it must be freshly ignited.)

Alcohol, redistilled over sodium or potassium hydroxide. This must be neutral: 5 cc. should only slightly alter the color of 50 cc. of water containing a little methyl red which has been adjusted previously with very dilute alkali to an intermediate orange color.

Methyl red, a 0.004 per cent solution in 50 per cent alcohol.

Standard acetate mixture. Dilute 50 cc. of 2 N acetic acid, mixed with 35 cc. of 2 N carbonate-free sodium hydroxide to 100 cc. with water.

Ammonia solution. 2.5 volumes of concentrated ammonia solution diluted with water to 100 volumes.

Paper pulp. Shake one 15 cm. ashless filter paper (e.g., Schleicher and

Schüll, No. 589, black ribbon) with 200 cc. of water in a stoppered bottle until the paper is thoroughly broken up.

Hydrochloric acid, 0.1 N.

Sodium hydroxide, 0.1 N.

Procedure

To an amount of urine containing from 2 to 7 mg. of inorganic phosphorus, in a large (20 by 200 mm.) lipped test-tube, add enough water to make about 10 cc. If more than 10 cc. of urine are taken proportionally larger quantities of all reagents must be used. If more than 20 cc. of urine are used, the prescribed fifteen minutes of shaking may be too short to effect complete precipitation. With urines as dilute as this, the method is hardly more accurate than the colorimetric micromethods described below. For each 10 cc. of urine solution, 1 cc. of magnesium citrate mixture and 2 cc. of concentrated ammonia solution are added. The tube is shaken until crystallization begins and then gently, but continuously, for fifteen minutes longer. During the latter period if the mixture is merely kept in motion precipitation is greatly accelerated; if the solution is left motionless twelve hours may not suffice for complete precipitation. The mixture is filtered with very gentle suction by means of a special filtration tube.

The special filtration tube is made from a piece of glass tubing about 8 mm. in internal diameter and 120 mm. long, flanged at the upper end, and at the lower end contracted to a bore of 2 mm. The capacity of such a tube should exceed 5 cc. The tube is supported by a rubber stopper in the neck of a suction flask large enough to hold a test-tube with a capacity of about 50 cc. A sufficient amount of paper pulp to make a mat thick enough to cover the bottom of the tube is introduced by means of a glass tube and packed by suction.

The precipitate and the tube in which it formed are washed first with 10 cc. of 2.5 per cent ammonia solution and then with four 5-cc. portions of alcohol, without any attempt to dislodge the part of the precipitate that adheres to the walls of the test-tube. Throughout this procedure the filtration tube should drain into a large test-tube set inside the suction-flask in order to keep the flask dry. It is well to wash out the receiving test-tube between the second and third alcohol washings. Remove the filtration tube from the suction-flask and support it by a clamp with its lower end in the mouth of a 100-cc. Erlenmeyer flask. With a calibrated pipette or micro-burette introduce into the test-tube where the phosphate was precipitated 0.1 N hydrochloric acid, 1 cc. at a time, until, on shaking, the precipitate which still adheres

to the walls dissolves completely. The resulting solution is poured into the filtration tube. By means of a stiff, sharpened, nichrome wire, precipitate and paper mat are now poked through the 2-mm. hole of the filtration tube into the Erlenmeyer flask. The test tube and filtration tube are rinsed with 2 cc. of methyl red solution followed by 13 cc. of of water. To the contents of the flask 0.1 N hydrochloric acid is added from a burette, 1 cc. at a time, until the solution remains red after it has been shaken thoroughly. At least 0.5 cc. of hydrochloric acid in excess of the amount required to decompose the precipitate should be added The solution is poured back and fourth between the test tube and flask until the precipitate is completely dissolved; then the few drops still remaining in the test tube are rinsed into the flask with 5 cc. of water.

The solution is now titrated with 0.1 N sodium hydroxide from a micro burette graduated into 0.01 or 0.02 cc. divisions. The alkali is added until the color begins to change, then the titration is completed by the introduction of 0.01 cc. of alkali at a time until the last addition causes the unknown solution to become definitely yellower than the standard. The volume of solution in the flask at the end of the titration should not be much greater than 25 cc.

Calculation

$$1.55\,(A - B - C) = \text{milligrams of phosphate P in sample.}$$

$$1.55\,(A - B - C) = \text{grams of phosphate P per liter of urine.}$$

$$\frac{50\,(A - B - C)}{V} = \text{millimoles of phosphate per liter of urine.}$$

A = cubic centimeters of 0.1 N hydrochloric acid, B = cubic centimeters of 0.1 N sodium hydroxide, used in the titration, C = value of $A - B$ found in a blank determination on the reagents; V = the cubic centimeters of urine taken for analysis.

INORGANIC PHOSPHATE IN URINE. TISDALL'S COLORIMETRIC METHOD (53)[3]

Reagents

The same as for the Tisdall method applied to determination of inorganic phosphate in serum (p. 875).

[3] Tisdall described the application of his method only to serum. However Dr. Alma Hiller (unpublished results) has found that, as above outlined, it is entirely satisfactory for urine, and in fact preferable to other colorimetric methods that were tried.

Procedure

The urine is well mixed to bring particles of insoluble phosphates into uniform suspension. One volume of urine (1 to 5 cc.) is mixed in a volumetric flask with approximately 4 volumes of water, and sufficient 10 per cent trichloroacetic acid is added to make the total volume 10-fold that of the sample. After ten minutes the solution, if not clear, is filtered.

One cubic centimeter of the filtrate is measured into a 15-cc. graduated centrifuge tube and diluted to 6 cc. with water. Two cubic centimeters of strychnine molybdate solution are added. The procedure from this point is the same as in the analysis of serum, outlined on page 875.

Calculation

$$\frac{0.5\ S}{U} = \text{grams of inorganic phosphorus per liter of urine.}$$

$$16.1\ S = \text{millimoles of phosphate per liter of urine.}$$

The above formulae hold when the solution of redissolved strychnine phosphomolybdate of the unknown is made up to 100 cc. When the unknown is made up to 50 cc. the results are divided by 2. If only half the redissolved precipitate is used, the result is multiplied by 2. If a urine is very low in phosphate content it may be desirable to take 2 cc. or more of the trichloroacetic acid filtrate for analysis instead of 1 cc. In this case the result calculated as above outlined is divided by the number of cubic centimeters of filtrate used.

To cover these conditions the usual formulae given above are expanded into the following:

$$\frac{0.5\ S}{U} \times \frac{1}{F} \times \frac{1}{P} \times \frac{v}{100} = \text{grams of inorganic phosphorus per liter of urine.}$$

$$\frac{16.1\ S}{U} \times \frac{1}{F} \times \frac{1}{P} \times \frac{v}{100} = \text{millimoles of phosphate per liter of urine.}$$

S = reading of standard, U = reading of unknown, F = cubic centimeters of trichloroacetic acid filtrate used for analysis, P = fraction of redissolved precipitate used for colorimetric determination, v = volume to which the final colored solution is made.

TOTAL PHOSPHORUS IN URINE

The total phosphorus may be obtained by ashing the urine by either the wet or dry method, described on pages 69 to 70, and determining the inorganic phosphate by any of the above described methods. Presumably, since all the known physiological organic phosphorus compounds are hydrolyzable phosphoric acid combinations, ashing is unnecessary, and boiling the urine with dilute mineral acid would be sufficient to change all the phosphorus into free phosphoric acid.

PHOSPHORUS IN FECES

Part of the phosphorus is in organic combination. It is necessary to determine total and inorganic separately, if values for both organic and inorganic phosphates are desired. Ordinarily only total phosphorus is determined.

The phosphorus content of the feces varies greatly, so that it is difficult to predict the size of samples to take in order to obtain optimum amounts of PO_4 for the type of analysis used, unless one has knowledge of the regime of the subject. A gram of phosphorus in 50 grams of dried stool may be assumed for purposes of calculating samples, if data are not available to estimate more closely for the subject under examination.

INORGANIC PHOSPHATE

0.5 to 1 gram of dried, pulverized stool (p. 78) is transferred to a 100 cc. measuring flask and suspended in a few cubic centimeters of water. A few cubic centimeters of 5 per cent trichloroacetic acid are added to coagulate the proteins, and then enough to fill the flask to the mark. The mixture is thoroughly shaken, and is filtered.

An aliquot of 10 or 20 cc., containing 1 to 4 mg. of inorganic P, may be diluted to 60 cc., and treated with strychnine molybdate solution, for gravimetric determination of PO_4 by the Embden method described on page 873 for blood filtrates.

It is probable also that, with samples of about 2 cc., the Tisdall colorimetric procedure, described on page 875, could be used.

TOTAL PHOSPHORUS IN FECES. GRAVIMETRIC MAGNESIUM PYROPHOSPHATE METHOD

Two grams of dried feces may be ashed by either the Neumann or the Stolte method (pp. 69 and 70). Or 25 cc. of the stool suspension in 25 per cent sulfuric acid, described on page 78, may be concentrated in a

Kjeldahl flask and wet ashed with the addition of nitric acid or hydrogen peroxide. The acid solution of fecal ash is diluted to 50 to 60 cc. If a wet ashing method has been used the solution is boiled to transform back into orthophosphoric acid any portion that may have been changed into metaphosphoric. Magnesium citrate is then added, as described on page 859 for urine analysis. After the magnesium solution is added (not before, or calcium phosphate would precipitate) enough concentrated ammonia solution is added to neutralize the acid present, then 20 cc. additional ammonia. The rest of the analysis is carried out as described for urine.

TOTAL PHOSPHORUS IN FECES. GRAVIMETRIC STRYCHNINE MOLYBDATE METHOD

This is an application of the Embden-Fetter technique for total phosphorus in blood. Sufficient of the solution of fecal ash to contain 0.5 to 4 mg. of phosphorus (the P in the ash of about 0.1 gram of dry feces, or of 2 cc. of the stool suspension in 25 per cent sulfuric acid described on page 78 will ordinarily fall within this range) is neutralized to brom phenol blue with concentrated ammonia solution, and is then slightly acidified and diluted to 60 cc. volume. The PO_4 is precipitated with 20 20 cc. of strychnine molybdate solution, and is determined as described on page 873 for inorganic phosphate in blood filtrates.

For the preliminary ashing one may find it convenient to apply the technique described on page 880 for total blood phosphorus.

TOTAL PHOSPHORUS IN FECES. TITRIMETRIC URANIUM METHOD

The ash solution is prepared as described above, for the gravimetric magnesium pyrophosphate method. One dop of phenol red solution is added and the acid in the solution is neutralized with concentrated ammonia water. If much acid is present the solution should be cooled under the tap occasionally during the addition of the ammonia in portions. About 3 cc. of ammonia will be required for each cc. of concentrated sulfuric acid present. The solution in the presence of much strong acid, is at first rose colored. As ammonia is added it assumes the usual yellow color of dilute acid solutions, and when excess ammonia is present the alkaline red color of the indicator appears. Enough glacial acetic acid is added, a drop at a time, to turn the solution acid to the phenol red. The solution is then diluted to 50 cc. and the titration is carried out as described for urine.

TOTAL PHOSPHORUS IN FECES. TITRIMETRIC PHOSPHOMOLYBDATE METHOD OF SHOHL AND BROWN[4]

The preliminary treatment of the feces is designed to permit the determination of both phosphorus and calcium. Dry ashing has been preferred to digestion with sulfuric and nitric or hydrochloric and nitric acids. The ash is dissolved in nitric acid and neutralized, and the phosphorus precipitated as ammonium phosphomolybdate. Formaldehyde and an excess of sodium hydroxide are added. The excess is titrated with acid and the phosphomolybdate estimated by difference.

Reagents

Concentrated nitric acid.

Concentrated ammonium hydroxide solution.

60 per cent ammonium nitrate solution.

Molybdate solution. Dissolve 100 grams of molybdic acid anhydride (MoO_3) in 144 cc. of strong ammonia water (specific gravity 0.90) and 271 cc. of water. Slowly and with constant stirring pour the solution thus obtained into a nitric acid solution which has been prepared by mixing 489 cc. of concentrated nitric acid (specific gravity 1.42) with 1148 cc. of water. Keep the mixture in a warm place for several days, until a portion of it when heated to 40°C. deposits no yellow ammonium phosphomolybdate. Decant the solution from any sediment which has formed and preserve it in glass stoppered bottles. When the solution is required for phosphate analyses 5 cc. of concentrated nitric acid should be added to 100 cc. of the stock molybdate solution.

One per cent potassium nitrate solution (neutralized to thymol blue, if necessary, with sodium hydroxide).

0.04 per cent solution of thymol blue and *0.05 per cent methyl red* (see table 68, p. 812).

Forty per cent formaldehyde, "reagent" grade (neutralized to thymol blue, if necessary, with sodium hydroxide or hydrochloric acid).

Boiled and cooled distilled water, neutral to thymol blue, pH 9 for washing.

0.419 N sodium hydroxide (1 cc. of this solution is equivalent to 0.5 mg. of P). 419 cc. of 1 N solution are diluted to 1 liter.

[4] A. T. Shohl and H. B. Brown, personal communication.

For discussion of the principle of the ammonium phosphomolybdate titration, see page 851.

0.419 nitric acid.

The alkali must be prepared from carbonate free sodium hydroxide and standardized by titration against standard acid, using thymol blue at its alkaline end-point as indicator. The phosphorus equivalent per cc. of the alkali must be confirmed by analyses of standard phosphate solution.

Procedure

Preliminary preparation of stool. The stool is treated with alcohol, dried, pulverized and mixed by sieving as described on page 78.

Ashing of stool. From 0.5 to 4 grams of dried, pulverized feces, accurately weighed, are placed in a platinum crucible. The mixture is then ashed, either slowly below red heat (about 400°C.) in an electric oven, or by the Stolte method (see p. 70).

The ash is treated with 3 to 5 cc. of water and about 1 cc. of concentrated nitric acid and heated, if necessary, until it is practically dissolved. It is then filtered through an ash-free filter into a 100 cc. volumetric flask, and crucible and filter paper are carefully washed into the flask with four or five 5-cc. portions of hot water. To the filtrate after it has cooled are added 3 drops of methyl red and concentrated ammonium hydroxide is introduced, drop by drop with constant shaking, until the indicator becomes a faint definite pink (an excess of ammonium causes precipitation of calcium salts). The solution in the flask is now cooled and diluted to volume with water.

Precipitation of phosphomolybdate. An aliquot of the filtrate containing 10 to 15 mg. of P (usually about 25 cc.) is transferred to a 250-cc. wide-mouthed Erlenmeyer flask or beaker. Twenty-five cubic centimeters of 60 per cent ammonium nitrate and water to make 100 cc. are added. The flask is heated on a water bath to a temperature of 60 to 65° (not higher), and 30 to 40 cc. of molybdate solution are added. The mixture is well stirred and allowed to stand about fifteen minutes at 60° to 65°. It is then filtered at once through a small ash-free filter, or better, by suction through a small Caldwell crucible lined with a layer of specially prepared asbestos $\frac{1}{8}$ inch thick.[5] The precipitate in the flask

[5] The Caldwell crucible is similar to a Gooch except that the perforated plate at the bottom is separate and detachable from the remainder of the crucible.

A suitable quality of asbestos is essential. It can be prepared by shredding or cutting asbestos into small pieces, covering them with dilute hydrochloric acid (1 part concen-

is washed twice, by decantation, with 25-cc. portions of 1 per cent potassium nitrate. The precipitate is stirred up well each time and then allowed to settle before the washings are poured through the filter. The precipitate is transferred to the filter and washed with 1 per cent potassium nitrate until two fillings of the filter (collected separately) do not greatly diminish the color produced with phenolphthalein by 1 drop of the standard alkali.

Titration of the phosphomolybdate. Precipitate, asbestos pad, and perforated disc of filter are transferred back to the original flask or beaker with about 25 cc. of water, which is also used to wash the filter. The precipitate is dissolved by the addition of a small excess of 0.419 N sodium hydroxide, measured from a burette. Two or 3 cc. more alkali are added than is required to dissolve completely the yellow precipitate. The asbestos is removed by filtration through a Gooch crucible and washed with boiled and cooled neutral water. This gives a clear solution and makes the end point of the subsequent titration sharper than with the asbestos present. The solution is diluted to about 100 cc. with boiled and cooled neutral water and a few drops of thymol blue are introduced. The solution should be blue, indicating the presence of an excess of sodium hydroxide. Five cubic centimeters of neutral formaldehyde are added. If the solution does not remain blue more alkali must be added at this point. The solution is then titrated to the neutral point with 0.419 N nitric acid.

Calculation

$$0.5\ (\text{Alk.} - \text{Ac.} - \text{B}) = \text{milligrams of P in sample titrated.}$$

$$0.01615\ (\text{Alk.} - \text{Ac.} - \text{B}) = \text{millimoles of phosphate in sample.}$$

Alk. = cubic centimeters of alkali solution used in the titration, Ac. = cubic centimeters acid used, B = value of (Alk − Ac) found in a blank analysis.

---

trated HCl to 3 parts water) and heating the mixture on a boiling water bath for five hours. The asbestos is then filtered off in a Gooch and washed free of acid. It is then covered with 5 per cent sodium hydroxide and heated as before for three hours. It is then filtered again and washed first with water, then with dilute acid and finally with water again until the acid has been removed. The finer particles are removed by decantation, leaving moderately coarse asbestos for use. In laying the asbestos in the crucible suction must not be applied to the filter until almost all the water has run out.

To estimate the P content of the stool of an entire period the phosphorus found in the sample titrated is multiplied by the factor:

$$\frac{\text{weight of stool}}{\text{weight of portion ashed}} \times \frac{100}{\text{cubic centimeters of ash solution titrated}}$$

TOTAL PHOSPHORUS IN FECES. TISDALL'S COLORIMETRIC METHOD

A portion of solution of fecal ash is neutralized with ammonia, then *slightly* acidified with nitric acid, and brought to such a volume that the phosphorus content is between 0.03 and 0.15 mg. per cubic centimeter. A portion of 1 cc. is then diluted to 6 cc. in a 15-cc. centrifuge tube. Two cubic centimeters of strychnine molybdate solution are added, and the phosphate content is determined as described for inorganic phosphate in serum on page 875.

Phosphorus in Blood

PRECAUTIONS IN HANDLING BLOOD FOR PHOSPHORUS DETERMINATIONS

The determination most frequently required in clinical work is the inorganic phosphorus of the plasma, normally only about 3 to 5 mg. of P per 100 cc. In the cells there is a relatively huge supply of organic phosphorus (50 to 100 mg. per 100 cc.). The phosphoric acid of this fraction is extremely labile. If hemolysis is prevented in freshly drawn whole blood, the organic acid-soluble fraction may increase for two or three hours by esterification at the expense of the inorganic phosphorus. Later the reaction reverses, and it is immediately reversed by hemolysis, the organic acid-soluble phosphorus hydrolyzing rapidly to inorganic.

If *whole blood* or *cells* are to be analyzed for inorganic or acid-soluble phosphorus, the proteins should be precipitated with an acid coagulant, without previous laking of the cells (23, 39), in order to prevent hydrolysis of organic phosphoric acid esters. The determination of inorganic phosphate in the filtrate must be made by a method which avoids heat, and should be carried out soon after the filtrate is obtained, because allowing the acid solution to stand, even at room temperature, may cause a slow acid hydrolysis of the organic phosphoric esters.

When the plasma or serum phosphate is to be determined, the blood should be centrifuged quickly after it is drawn, and hemolysis must be prevented, in order to avoid spontaneous hydrolysis of organic cell phosphorus into inorganic. There is so little, if any, acid-soluble organic phosphorus in the plasma, however, that after the proteins have been precipitated the

plasma filtrate may even be heated with dilute sulfuric acid without significantly increasing the inorganic phosphate content (6).

INORGANIC PHOSPHATE OF SERUM, PLASMA OR BLOOD, GRAVIMETRIC EMBDEN-FETTER METHOD (20, 21)

The PO_4 is precipitated as strychnine molybdophosphate, which is dried at 110° and weighed. One milligram of P yields 89.3 mg. of precipitate. (For discussion of the method, see p. 849.)

To obtain a quantitative precipitate of this constant composition it is necessary that in all analyses 1 volume of strychnine molybdate solution be added to 3 volumes of phosphate solution, and that the phosphate solution at the time of the addition shall not contain mineral acid in more than 0.5 N, nor alkali in more than 2 N concentration (20, 49). The maximum amount of phosphorus precipitable per cubic centimeter of strychnine molybdate solution is 0.2 mg. If these conditions are observed, the method can be applied over a wide range of volumes and amounts of phosphate; e.g., in this analysis 20 cc. of strychnine molybdate solution are added to 60 cc. of blood filtrate, while in Tisdall's method, described next, the respective volumes are 2 and 6 cc.

Reagents

Molybdate solution. Fifty grams of ammonium molybdate are dissolved in warm water and made up to 150 cc. The solution is then poured into 450 cc. of a nitric acid solution which has been made by mixing 2 volumes of concentrated nitric acid (specific gravity 1.42) with 1 volume of water.

Strychnine nitrate solution. Three grams of strychnine nitrate are made up to 200 cc. with water.

Strychnine molybdate solution. One volume of strychnine nitrate solution is poured with stirring into 3 volumes of the above molybdate solution. This strychnine molybdate solution is prepared fresh for each analysis.

Trichloroacetic acid, 10 per cent solution. The solution is tested for phosphates by blank analysis. If any precipitate is obtained with strychnine molybdate, the trichloroacetic acid is purified by distillation *in vacuo.*

Procedure

Five cubic centimeters of blood, plasma, or serum are measured into a 50-cc. flask containing 25 cc. of 10 per cent trichloroacetic acid.[6] The

[6] Fetter laked the whole blood before precipitating the proteins. As stated in the preceding discussion of the treatment of blood, this procedure is likely to cause error from hydrolysis of organic phosphoric acid esters.

flask is filled to the mark with water and, after about 15 minutes, the contents are filtered. Twenty-five cubic centimeters of the filtrate, equivalent to 2.5 cc. of blood or plasma, are diluted to 60 cc., and 20 cc. of the strychnine molybdate solution are added. The mixture is allowed to stand at room temperature for one hour, with occasional shaking, for the precipitation to become complete. The contents of the flask are filtered through a small Gooch crucible or a filter tube of the type described by Shohl (fig. 85, p. 742), which has previously been dried at 110°, cooled in a desiccator, and weighed. It is important to use only gentle suction during filtration. A mercury air valve (fig. 45, p. 302) regulating the suction at about 100 mm. of mercury, may be used to advantage. Particles of precipitate which adhere to the flask are rinsed into the Gooch crucible with 25 cc. of ice-cold 5-fold diluted strychnine molybdate solution. The precipitate is then washed with ice-cold water until the washings are no longer acid. During the washing, as soon as one portion of fluid has been drawn through the crucible another portion is added, or else the suction is stopped. If suction is continued without fluid in the crucible, the precipitate becomes divided by cracks, through which subsequent portions of wash water will run, without making contact with most of the precipitate. The crucible is dried for an hour at 110°, cooled in a desiccator, and weighed.

Calculation

$$0.0112\ W = \text{milligrams of phosphate P in sample.}$$

$$\frac{1.12\ W}{V} = \text{milligrams of phosphate P per 100 cc. of blood or plasma.}$$

$$\frac{0.361\ W}{V} = \text{millimoles of phosphate per liter of blood or plasma.}$$

W = weight of precipitate in milligrams, V = cubic centimeters of blood or plasma represented in the sample.

When, as above directed, filtrate equivalent to a blood sample of 2.5 cc. is used, the calculations simplify to:

$$0.448\ W = \text{milligrams of phosphate P per 100 cc. of blood or plasma.}$$

$$0.1442\ W = \text{millimoles of phosphate per liter.}$$

Note: The weight of precipitate obtained in analysis of normal plasma as above described is usually 8 to 12 mg. When the technique of ordinary

macro gravimetric analysis is used, the accuracy of results could doubtless be increased by using larger blood samples. The final volume and conditions at which the precipitation is made, however, should not be altered as, according to Embden, the phosphorus content of the precipitate is constant at the figure given only under the prescribed conditions of precipitation Any amount of phosphorus up to 4 mg. may be determined by this method.

INORGANIC PHOSPHATE OF SERUM OR BLOOD. TISDALL'S COLORIMETRIC DETERMINATION ON EMBDEN PRECIPITATE (53)

Proteins are removed with trichloroacetic acid, phosphate is precipitated with strychnine molybdate, and the Mo^{vi} of the precipitate is reduced to a green product by means of ferrocyanide. (For discussion of the method in comparison with other colorimetric procedures, see p. 854.) Tisdall described the method only for serum, but there is no apparent reason why it can not be applied, like the Embden-Fetter method, to whole blood also.

Reagents

All the *reagents described above for the Embden-Fetter gravimetric method* are used. The trichloroacetic acid for the Tisdall serum analysis should be 6 per cent, however, instead of 10 per cent.

In addition, the following are required:

One per cent NaOH solution.

Twenty per cent potassium ferrocyanide. Twenty grams of $K_4Fe(CN)_6$ dissolved in water and made up to 100 cc.

Concentrated HCl solution.

Standard phosphate solution containing 5 mg. of P per 100 cc. The same stock solution, containing per liter 4.388 grams of KH_2PO_4, serves for this analysis as for the uranium titration. Five cubic centimeters of the stock solution are made up to 100 cc. with water.

Procedure

Precipitation of protein. **Two cc. of serum are transferred to a 15-cc. centrifuge tube and to this are added 10 cc. of a 6 per cent solution of trichloroacetic acid. The mixture is thoroughly mixed with the aid of a glass rod and allowed to stand for four minutes. It is then centrifuged for four to five minutes at about 1500 revolutions per minute and the supernatant fluid poured off.**

Of whole blood, 2 cc. may be precipitated with 10 cc. of 10 per cent trichloroacetic acid.

Precipitation of phosphorus with the strychnine molybdate reagent. Five cubic centimeters of the supernatant fluid are measured into an ordinary 15-cc. graduated centrifuge tube, the outside diameter of which is 6 to 7 mm. at the 0.1-cc. mark. Water is added to bring the volume to 6 cc., followed by 2 cc. of the strychnine molybdate reagent. This should be added drop by drop, and the tube shaken three or four times during the addition. The contents of the tube are then thoroughly mixed by holding the tube at the upper end and tapping the lower end with the finger to give it a circular motion. The contents are allowed to stand for 10 minutes, during which time they are thoroughly mixed twice as outlined above.

Washing of precipitate. After the ten minutes have elapsed the tube is centrifuged at 1500 revolutions per minute for three minutes, the supernatant fluid is poured off and the mouth of the tube wiped with a dry cloth. Three cubic centimeters of water are allowed to run down the sides of the tube to remove any adherent supernatant fluid. The residual supernatant fluid (about 0.1 cc.) is thoroughly mixed with the added water by tapping the lower end of the tube with the finger to give it a circular motion, while the precipitate is disturbed as little as possible. The mixture is centrifuged for one minute at 1500 revolutions per minute, the supernatant fluid is poured off and the above procedure repeated, making two washings in all.

Development of color. After the final supernatant fluid has been removed, 2 cc. of a 1 per cent solution of NaOH are added and the contents mixed with the aid of a glass rod. This causes all the precipitate to go into solution. Water is added to 10 cc. and the contents are transferred to a 100-cc. glass-stoppered volumetric flask. Traces of the solution remaining in the centrifuge tube are washed into the flask by means of two lots of 10 cc. of water, so that the total volume of fluid in the flask is 30 cc. Twenty cubic centimeters of a 20 per cent solution of potassium ferrocyanide are then added, followed by 10 cc. of concentrated HCl. The flask is inverted two or three times and allowed to stand ten minutes. Water is added to 100 cc., the contents are thoroughly mixed, and the color is read in the colorimeter against the standard.

Preparation of the standard. One cubic centimeter of the solution of KH_2PO_4 containing 5 mg. of P per 100 cc. is measured into a graduated centrifuge tube, which contains 5 cc. of water, and the con-

tents are thoroughly mixed. Two cubic centimeters of the strychnine molybdate reagent are then added drop by drop. This step, and the washing of the precipitate and the development of the color, are carried out at the same time and in the same manner with both the standard and the unknown.

The volume of the precipitate. The amount of precipitate obtained in the standard solution after it is centrifuged is almost exactly 0.1 cc. in volume. If the amount of precipitate obtained in the unknown is 0.15 cc. or more, its solution (in 1 per cent NaOH) should be made up to a definite volume in the centrifuge tube and an aliquot taken which would contain approximately 0.1 cc. of the precipitate. If the amount of precipitate obtained in the unknown is about one-half the amount in the standard, its solution should be made up to 5 cc. and transferred to a 50-cc. volumetric flask with the use of two lots of 5 cc. of water. In all the subsequent steps the volumes used should be halved.

Calculation

$$\frac{0.05\ S}{U} = \begin{cases}\text{milligrams of P in portion of redissolved precipitate} \\ \quad \text{used for colorimetric determination.}\end{cases}$$

$$\frac{6\ S}{U} = \text{milligrams of inorganic P per 100 cc. of blood or serum.}$$

$$\frac{1.93\ S}{U} = \begin{cases}\text{millimoles of inorganic phosphate} \\ \quad \text{per liter of blood or serum.}\end{cases}$$

The above formulae hold when the unknown colored solution is made up to 100 cc. When the unknown is made up to 50 cc. the result is divided by 2. If only half the redissolved strychnine phosphomolybdate of the unknown is used, the result is multiplied by 2.

S = reading of standard, U = reading of unknown.

INORGANIC PHOSPHATE OF SERUM OR PLASMA. DIRECT COLORIMETRIC METHOD OF BELL AND DOISY AS MODIFIED BY BENEDICT AND THEIS (6)

Because the color is developed in a highly acid solution with the aid of heat this method is not applicable to the analysis of whole blood filtrates. (For discussion of direct colorimetric methods, the factors which influence the results, and the precautions required, see page 855 to 857.)

Reagents

Molybdate reagent. To 20 grams of molybdic acid anhydride (MoO_3), strictly pure and free from ammonia, add 25 cc. of 5 N sodium hydroxide solution, and warm the mixture gently until the molybdic acid dissolves. Cool and dilute to 250 cc. Filter the reagent if necessary.

Molybdate sulfuric acid reagent. A small quantity of the molybdate reagent (enough for a few day's use only) is diluted with an equal volume of concentrated sulfuric acid as it is needed.

Hydroquinone-sulfite solution. Dissolve 15 grams of sodium bisulfite, $NaHSO_3$, and 0.5 gram of hydroquinone in water and dilute the mixture to 100 cc. Keep the solution stoppered to prevent oxidation by air.

A stock standard solution of potassium dihydrogen phosphate (1 cc. contains 1 mg. of phosphorus). This is the same solution, containing 4.388 grams of KH_2PO_4 per liter, described on p. 861 for uranium titration.

Standard phosphate solutions for use in blood analysis. Four standards are prepared by diluting 4, 3, 2, and 1 cc. portions of the stock solution to 200 cc. each with a 10 per cent solution of the same trichloroacetic acid used for precipitation of blood proteins. If a slight trace of phosphoric acid is present in the trichloroacetic acid, error from it is thus compensated. The above solutions contain, in the 5-cc. portions used for analyses, 0.10, 0.075, 0.05, and 0.025 mg. of phosphorus respectively.

Twenty per cent trichloroacetic acid. This is tested for phosphoric acid by boiling a 10-cc. portion for a few minutes to decompose most of the acid, and then adding 2 cc. of the strychnine molybdate solution described above for the Embden-Zetter method. If a significant amount of precipitate forms, the trichloroacetic acid is redistilled *in vacuo* before it is used for blood analyses.

Procedure

Pipette 2 cc. of serum into a 10-cc. volumetric flask containing a little water. Four cubic centimeters of 20 per cent trichloroacetic acid are added. Dilute the contents of the flask to the mark with water, mix and set aside for at least ten minutes. Filter the liquid through an ashless filter paper. Transfer 5 cc. of the filtrate, equivalent to 1 cc. of serum, to a test tube. Add 3 cc. of water, 1 cc. of the molybdate sulfuric acid reagent, 1 cc. of hydroquinone sulfite solution, and mix well. At the same time treat 5 cc. of the appropriate phosphate standard in a similar manner in another tube. (For normal serum the standard with 0.05 mg. of P is usually closest.) Stopper both tubes loosely and stand them in a

boiling water bath for ten minutes. Cool to room temperature and compare standard and unknown in the colorimeter.

Calculation

$$\frac{100\ D\ S}{U} = \text{milligrams of phosphate P per 100 cc. of serum.}$$

$$\frac{32.2\ D\ S}{U} = \text{millimoles of inorganic phosphate per liter of serum.}$$

S and U represent the readings of the standard and unknown respectively, and D the number of milligrams of phosphorus in the standard solution (D is ordinarily 0.05 mg.).

INORGANIC PHOSPHATE OF WHOLE BLOOD. DIRECT COLORIMETRIC METHOD. BRIGGS' MODIFICATION OF THE BELL-DOISY PROCEDURE (10, 5)

For discussion of principle and conditions, see page 855.

The Benedict-Theis procedure described above can not be used with the filtrate from whole blood, because it involves heating in acid solution, which would hydrolyze the abundant organic phosphates in the filtrate from the cells. The Briggs procedure does not yield so deep a color with the small amounts of phosphorus, but is carried out without heat. It can be used also for serum, but the Benedict-Theis method is preferable.

Reagents

Standard phosphate solution, containing 0.01 mg. of P per cubic centimeter. Two cubic centimeters of the stock solution, containing 1 mg. per cubic centimeter, described on page 861 for the uranium titration, are diluted to 200 cc. with a 10 per cent solution of the same trichloroacetic used to precipitate blood proteins in the analysis.

Ammonium molybdate solution. Dissolve 25 grams of ammonium molybdate in 300 cc. of water. When solution is complete add 75 cc. of concentrated sulfuric acid and make up to 200 cubic centimeters with water.

Hydroquinone solution. Dissolve 1 gram of hydroquinone in 100 cc. of water and add 1 drop of concentrated sulfuric acid to retard oxidation.

Twenty per cent sodium sulfite solution. Twenty grams of anhydrous sodium sulfite made up to 100 cc. with water. The solution must be kept well stoppered to avoid oxidation by the air, or must be made up fresh.

10 N sulfuric acid, 300 cc. of concentrated sulfuric acid diluted with water to 1 liter.

A 20 per cent solution of trichloroacetic acid, tested as for Benedict-Theis method.

Procedure

The proteins of 2 cc. of blood or plasma are precipitated at 10-cc. volume as described for the Benedict-Theis method.

Transfer 5 cc. of the filtrate, equivalent to 1 cc. of blood or plasma, to a 10-cc. volumetric flask. In another 10-cc. flask place 3 cc. of a standard potassium dihydrogen phosphate solution containing 0.03 mg. of phosphorus. To each flask add 1 cc. of the molybdate, 1 cc. of the hydroquinone and 1 cc. of the sulfite solutions, in the order named, and dilute to 10 cc. with water. After thirty minutes compare standard and unknown in the colorimeter.

Calculation is same as for Benedict-Theis method above.

TOTAL PHOSPHORUS OF BLOOD, SERUM, OR PLASMA. GRAVIMETRIC EMBDEN-FETTER METHOD (20, 21)

Reagents

Same as for Embden-Fetter method for inorganic phosphate (p. 873). In addition, concentrated nitric and sulfuric acids.

Procedure

Digestion of organic matter. This is done by Neumann's wet ashing method. One cubic centimeter of whole blood, or 2 cc. of serum or plasma, is pipetted into a hard glass or silica test tube or micro Kjeldahl flask of 75 to 100-cc. capacity, which has been previously cleaned by boiling concentrated sulfuric acid in it for twenty minutes. Five cubic centimeters of a mixture of equal volumes of concentrated sulfuric and nitric acids are added, and a piece of quartz or a glass bead to prevent bumping. The tube is heated with a micro burner until white fumes appear, and then for ten minutes longer. Just enough heat is applied to keep the tube filled with white fumes, but not enough to drive them out of the tube.

Precipitation of strychnine phosphomolybdate. The cooled digest is washed with water into a 250-cc. Erlenmeyer flask. Three drops of brom-phenol blue are added, then concentrated ammonia until the solution turns alkaline, and finally just enough normal sulfuric acid to change the color back to acid. The solution is cooled and made up to 60-cc. volume. Then 20 cc. of the strychnine molybdate solution are

added and the phosphate is determined as described above for Embden-Fetter inorganic phosphate.

TOTAL PHOSPHORUS OF BLOOD, SERUM, OR PLASMA. TISDALL'S COLORIMETRIC DETERMINATION ON EMBDEN PRECIPITATE (53)

Reagents

Same as for Tisdall determination of inorganic phosphate (p. 875), and in addition a mixture of equal parts of concentrated nitric and sulfuric acids.

Procedure

Digestion. The digestion is carried out as in the preceding method, except that only 0.5 cc. of whole blood, or 1 cc. of serum, is taken, and 2 cc. of the mixture of nitric and sulfuric acids. After digestion the residue is transferred to a 25-cc. measuring flask, where it is neutralized, as in the preceding method, and made up to volume.

Precipitation. A 10-cc. aliquot, representing 0.4 cc. of serum or 0.2 cc. of whole blood, is pipetted into a 15-cc. graduated centrifuge tube, and 3.3 cc. of strychnine molybdate solution are added from a graduated 5-cc. pipette.

The rest of the determination is carried through exactly as described above for inorganic phosphate.

Calculation

For whole blood, when samples = 0.2 cc. of blood

$$\frac{25\ S}{U} = \text{milligrams of total P per 100 cc.}$$

$$\frac{8.07\ S}{U} = \text{millimoles of total P per liter of blood.}$$

For serum, when sample = 0.4 cc. of serum

$$\frac{12.5\ S}{U} = \text{milligrams of total P per 100 cc. of serum.}$$

$$\frac{4.03\ S}{U} \quad \text{millimoles of total P per liter of serum.}$$

The above formulae hold when the unknown solution used for colorimetry is made up to 100 cc. If only half the redissolved strychnine phosphomolybdate is used, the result is multiplied by 2.

TOTAL PHOSPHORUS OF WHOLE BLOOD, SERUM, OR PLASMA. DIRECT COLORIMETRIC METHOD. WHITEHORN'S (55) MODIFICATION OF THE BELL-DOISY PROCEDURE[7]

The organic matter is destroyed by digestion with sulfuric and nitric acids, remaining traces of nitric acid are destroyed by sulfite, and the phosphoric acid is determined essentially according to the Benedict-Theis modification of the Bell-Doisy method.

Reagents

Molybdic acid. Five per cent ammonium molybdate in 2 N sulfuric acid. This keeps indefinitely.

Hydroquinone. Two per cent in water. This keeps several weeks if 2 to 4 drops of concentrated sulfuric acid per 100 cc. are added, and the solution is kept tightly stoppered.

Sodium sulfite. Twenty grams of anhydrous salt dissolved in water to make 100 cc. In tightly stoppered bottles it may keep for months, but it it is advisable to prepare a fresh supply every few weeks.

Standard phosphate solutions. 4.388 grams of pure KH_2PO_4 and 20 cc. of concentrated sulfuric acid are dissolved in water and diluted to a liter. The solution contains 1 milligram of P per cubic centimeter. The standard for use in blood analysis is prepared at intervals by diluting this strong standard 1 to 100 with water.

Procedure

Make 0.5 volume of cells, 1 volume of blood, or 5 volumes of plasma up to 10 volumes with water. Transfer 1 cc. of the mixture to a hard glass test tube, of 25 by 200-mm. dimensions, add a quartz or glass bead, 1 cc. of concentrated sulfuric acid, and 1 cc. of concentrated nitric acid. Digest the mixture over the naked flame of a micro burner for ten minutes after white fumes appear. Heat just enough to keep the tube nearly filled with fumes, but not so strongly as to drive fumes out of the tube.

Let the residue cool for three minutes. Add cautiously 2 cc. of 20 per cent sodium sulfite solution and another bead. Mix and heat the contents of the tube, with the burner at the same height as before, for two minutes. The heating should be stopped if white fumes appear, since

[7] Whitehorn published his method for lipoid phosphorus. Its applicability to total blood phosphorus has, however, been ascertained by Dr. Alma Hiller (unpublished results).

the higher boiling temperature of the salt-acid mixture may convert orthophosphoric into pyrophosphoric acid.[8]

After cooling a minute or more add 5 cc. of distilled water and cool the tube in cold water.

In a similar tube place 5 cc. of the standard phosphate, containing 0.01 mg. of P per cubic centimeter, then 1 cc. of concentrated sulfuric acid, and cool.

Add to each tube 2 cc. of acid molybdate solution, 2 cc. of sodium sulfite solution, and 1 cc. of hydroquinone solution, in the order given, shaking after each addition.

Transfer each solution to a 16 by 170-mm. test tube marked at 15, 20 and 25 cc., rinsing with water until the volume is 15 cc. Stopper with clean thumb and invert twice. Set in a boiling water bath for ten minutes. Cool at the tap. Add water to the standard up to the 25-cc. mark and mix. The unknown may be made up with water to 15, 20 or 25 cc., whichever gives a color approximately that of the standard. Compare the colors in a colorimeter with the standard set at 20 mm. A series of 4 or 5 unknowns can be carried through with one standard.

Calculation

$$\text{Milligrams of P per 100 cc.} = \frac{S}{U} \times 0.05 \times \frac{100}{V} \times \frac{D}{25} = \frac{0.2\ SD}{U\ V}$$

S indicates the reading of the standard, U of the unknown. V is the cubic centimeters of blood or plasma represented in the portion of filtrate taken for analysis (usually $V = 0.1$ cc. for blood, 0.5 for plasma). D represents the volume in cubic centimeters to which the unknown was diluted before the color reading.

ACID-SOLUBLE PHOSPHORUS IN CELLS OR WHOLE BLOOD

The cells or whole blood are mixed with 9 volumes of 10 per cent trichloroacetic acid. Portions of the filtrate are then digested and analyzed as described above for total phosphorus. Samples of the following volumes of filtrate are taken for the different methods of analysis.

Embden-Fetter gravimetric, 10 cc.
Tisdall, colorimetric, 5 cc. (for duplicates).

[8] Instead of nitric acid one may use 1 gram of potassium persulfate as oxidizing agent. The addition of and heating with 2 cc. of sulfite then becomes unnecessary.

Whitehorn, 0.5 cc. of cell filtrate or 1 cc. of whole blood filtrate.

The calculations are the same as for total phosphorus.

If the Tisdall method is applied to cell filtrate it will be desirable to use 5 cc. instead of 10 cc., of the 25 cc. digested solution, for the colorimetric analysis. In this case the results of the whole blood calculation formulae on p. 881 will be multiplied by 2.

LIPOID PHOSPHORUS OF SERUM OR PLASMA BY WET ASHING OF BLOOR'S EXTRACT

This method is applicable to the analysis of Bloor's alcohol-ether lipoid extract of blood, plasma or serum (see determination of lipoids, p. 495). The lipoids are extracted by alcohol and ether and the phosphorus content of the extract is determined by the total phosphorus method. Since the lipoids are precipitated with the proteins by trichloroacetic acid, lipoid phosphorus could also presumably be determined in an extract of the protein precipitate (17).

Reagents

Alcohol-ether mixture. Three volumes of redistilled alcohol and 1 volume of redistilled ether.

The other reagents are the same as those used for the determination of total phosphorus.

Extraction of lipoids

Into a glass-stoppered 100-cc. volumetric flask pour about 75 cc. of the alcohol-ether mixture, whirl the flask a few times and, as the contents rotate, add in a fine stream from a pipette 5 cc. of whole blood or plasma. Immerse the flask in hot water, continue to rotate it gently until the mixture boils, then cool in running water, dilute it to the mark with alcohol-ether, mix and filter. Cover the funnel with a watch-glass during filtration and stopper the bottle containing the filtrate as soon as it is collected.

Determination of phosphorus in lipoid extract

The phosphorus content of the extract is determined by wet ashing and gravimetric or colorimetric analysis, by the methods described above for total phosphorus in blood. Portions of the extract for the different analyses are taken of the following volumes.

Embden-Fetter method, **40 cc.**

Tisdall method, **20 cc. (for duplicates).**

Whitehorn method, **10 cc.**

The portion of extract is placed in the test tube or Kjeldahl flask in which the digestion is to be carried out, a glass bead is added, and the solution is evaporated in a water bath to dryness. The residue is then digested with sulfuric and nitric acids, as described for total blood phosphorus by the above methods, and the remainder of the analysis, including the calculation, is carried out as described for total phosphorus.

COLORIMETRIC DETERMINATION OF LIPOID PHOSPHORUS OF BLOOD, PLASMA, OR SERUM WITHOUT OXIDATION OF ORGANIC SUBSTANCES. HARNES (27)

The phospholipoids are extracted with chloroform. They are then hydrolyzed by the action of the sulfuric acid in Briggs' molybdate solution itself, while at the same time the blue color of reduced molybdate is developed.

Reagents

Chloroform.

Molybdate solution. Dissolve 25 grams of ammonium molybdate in 300 cc. of water, add 75 cc. of concentrated sulfuric acid and dilute the whole with 125 cc. of water.

Hydroquinone solution. Dissolve 5 grams of hydroquinone and 25 grams of potassium bisulfite in 500 cc. of water.

Phosphate standards. A solution containing 0.01 mg. of P per cc. is made by 100-fold dilution of the stock KH_2PO_4 solution described on p. 861.

Procedure

One cubic centimeter of whole blood, plasma or serum is spread on a strip of fat-free filter paper about 4 by 17 cm. in size. The blood on the paper is dried in an electric oven at 50°. The strip is then placed in a Folin sugar tube containing about 4 cc. of chloroform and fitted with a condenser, composed of a test tube through which water is allowed to syphon (see figure 91). In this apparatus the chloroform is refluxed on a water bath at 75°C. for three hours. When the extraction is complete the chloroform is transferred to a 50 cc. volumetric flask, in which are also placed 20 cc. of water, 3 cc. of molybdate solution, and 3 cc. of hydroquinone solution. The unstoppered flask is immersed in boiling water for 30 minutes to drive off the chloroform and cause complete color development. A measured volume of the phosphate standard, usually 10 cc. containing 0.1 mg. of P, is at the same time

diluted to 20 cc. with water in another 50 cc. flask, and heated with molybdate and hydroquinone. Unknown and standard are cooled, made up to 50 cc. each, and compared in a colorimeter.

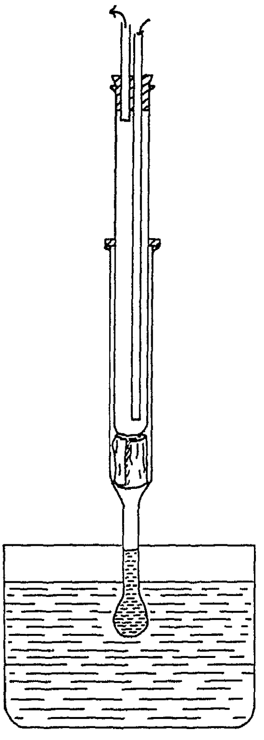


FIG. 91. Harnes' extraction and digestion tube for the determination of lipoid phosphorus

Calculation

$$\text{Milligrams lipoid P per 100 cc.} = \frac{100\,n\,S}{U}$$

S = reading of standard, U = reading of unknown, n = milligrams of P present in the standard. Since the standard contains 0.01 mg. of P per cubic centimeter, when the usual volume of 10 cc. is taken n = 0.1.

Bloor has proposed a method for the determination of phospholipoids by an adaptation of his oxidative titration. This has been described in the chapter on the determination of lipoids (p. 502).

BIBLIOGRAPHY

1. Association of Official Agricultural Chemists. Methods of Analysis. Washington, 1925.
2. Atkins, W. R. G., and Wilson, G. W.: On the colorimetric estimation of minute amounts of compounds of silicon, phosphorus, and arsenic. *Biochem. J.*, 1926, **20**, 1223.
3. Bang, I.: Methodologische Notizen. *Biochem. Z.*, 1911, **32**, 445.
4. Baumann, E. J.: On the estimation of organic phosphorus. *J. Biol. Chem.*, 1924, **59**, 667.
5. Bell, R. D., and Doisy, E. A.: Rapid colorimetric methods for the determination of phosphorus in urine and blood. *J. Biol. Chem.*, 1920, **44**, 55.
6. Benedict, S. R., and Theis, R. C.: A modification of the molybdic method for the determination of inorganic phosphorus in serum. *J. Biol. Chem.*, 1924, **61**, 63.
7. Bloor, W. R.: A method for the determination of "lecithin" in small amounts of blood. *J. Biol. Chem.*, 1915, **22**, 133.
8. Bloor, W. R.: Studies on blood fat. II. Fat absorption and the blood lipoids. *J. Biol. Chem.*, 1915, **23**, 317.
   The blood lipoids in nephritis. *J. Biol. Chem.*, 1917, **31**, 575.
   Methods for the determination of phosphoric acid in small amounts of blood. *J. Biol. Chem.*, 1918, **36**, 33.
9. Bloor, W. R., and Farrington, E. D.: Blood phosphates in the lipemia produced by experimental anemia in rabbits. *J. Biol. Chem.*, 1920–21, **45**, 171.
10. Briggs, A. P.: A modification of the Bell-Doisy phosphate method. *J. Biol. Chem.*, 1922, **53**, 13.
11. Briggs, A. P.: Some applications of the colorimetric phosphate method. *J. Biol. Chem.*, 1924, **59**, 255.
12. Buell, M. V.: On the phosphorus compounds in normal blood. *J. Biol. Chem.*, 1923, **56**, 97.
13. Denigès, G.: Nouveau mode de dosage volumétrique de molybdène. *Compt. rend. Acad. Sci.*, 1927, **184**, 330.
14. Denigès, G.: Détermination quantitative des plus faibles quantités de phosphates dans les produits biologiques par la méthode céruléomolybdique. *Compt. rend. soc. biol.*, 1921, **17**, 1974.
15. Denigès, G.: Micro-dosage immédiat de l'ion phosphorique, par céruléomolybdimétrie, dans les liquides de l'organisme, les eaux naturelles, les boissons fermentés, les matières agricoles, etc. *Compt. rend. Acad. Sci.*, 1928, **186**, 318.
16. Denis, W., and von Meysenbug, L.: Note on a possible source of error in the Bell-Doisy method for the determination of phosphates in blood plasma. *J. Biol. Chem.*, 1922, **52**, 1.
17. De Toni, G. M.: The colorimetric estimation of cholesterol and lecithin in blood in connection with Folin and Wu's system of blood analysis. *J. Biol. Chem.*, 1926, **70**, 207.
18. Dienes, L.: Further studies in the determination of calcium, magnesium, and phosphorus in animal substances. *J. Biol. Chen.*, 1924, **61**, 77.
19. Du Parc, L., and Rogovine, E.: Sur un nouvel indicateur pour le dosage volumétrique de l'acide phosphorique. *Helv. Chim. Acta.*, 1928, **11**, 598.

20. EMBDEN, G.: Eine gravimetrische Bestimmingsmethode für kleine Phosphorsäuremengen. *Z. physiol. Chem.*, 1921, **113**, 138.

21. FETTER, W. J.: Gravimetric estimation of phosphates of the blood. Phosphates in nephritis. *Arch. Int. Med.*, 1923, **31**, 413.

22. FISKE, C. H.: The determination of inorganic phosphate in urine by alkalimetric titration. *J. Biol. Chem.*, 1921, **46**, 285.

23. FISKE, C. H., AND SUBBAROW, Y.: The colorimetric determination of phosphorus. *J. Biol. Chem.*, 1925, **66**, 375.

24. GILBERT, B. E., AND SMITH, J. B.: The ceruleomolybdate estimation of phosphate phosphorus. *J. Biol. Chem.*, 1927, **74**, 223.

25. GREENWALD, I.: On the phosphorus content of the blood of normal and parathyroidectomized dogs. *J. Biol. Chem.*, 1913, **14**, 369.
The estimation of lipoid and acid soluble phosphorus in small amounts of serum. *J. Biol. Chem.*, 1915, **21**, 29.

26. HAMMARSTEN, GRETA: A simple method for the titration of ammonium phosphomolybdate. *Compt. rend. lab. Carlsberg*, 1927, **17**, No. 5.

27. HARNES, A. R.: The colorimetric determination of lipoid phosphorus in blood. *J. Biol. Chem.*, 1928, **77**, 405.

28. HUNDESHAGEN, F.: Analytische Studien über die Phosphordodekamolybdänsäure, die Bedingungen ihrer Bildung, und ihrer Abscheidung als Ammoniumsalz. *Z. anal. Chem.*, 1889, **28**, 141.

29. JAVILLIER, M., AND DJELATIDÈS, D.: Sur un méthode de microdosage volumétríque du phosphore et son application à l'analyse biologique. *Bull. soc. biol. chim.*, 1928, **10**, 342.

30. JONES, W.: The admissibility of ammonium magnesium phosphate as a form in which to weigh phosphoric acid. *J. Biol. Chem.*, 1916, **25**, 87.

31. KOBER, P. A., AND EGERER, G.: Nephelometric estimation of phosphorus. *J. Am. Chem. soc.*, 1915, **37**, 2373.

32. KUHN, R.: Die Microbestimmung der Phosphorsäure. *Z. f. physiol. Chem.*, 1923, **129**, 64.

33. KUTTNER, T., AND, COHEN, H.: Microcolorimetric studies. I. A molybdic acid, stannous chloride reagent. The micro-estimation of calcium and phosphate in pus, plasma, and spinal fluid. *J. Biol. Chem.*, 1927, **75**, 517.

34. LEIBOFF, S. L.: The reduction of phosphomolybdic acid by monomethyl-*p*-amino phenol and its use in the quantitative determination of phosphorus. *J. Lab. Clin. Med.*, 1931, **16**, 495.

35. LIEB, H.: Pregl's Quantitative Organische Microanalyse, Berlin, 1917, p. 133.

36. LORENZ, N. VON.: Eine Methode zur Phosphorsäurebestimmung in Düngern, Boden, und Aschen. Die landwirtschaftlichen Versuchstationen, 1901, **51**, 183; 1907, **46**, 192.

37. MALOT, C.: Maasanalytische Bestimmung der Phosphorsaeure durch Uraniumitrat. *Chem. Centr.*, 1887, **18**, 873.

38. MATHISON, G. C.: The estimation of phosphorus in urine. *Biochem. J.*, 1909, **4**, 233.

39. MARTLAND, M., AND ROBISON, R.: Note on the estimation of phosphorus in blood. *Biochem. J.*, 1924, **18**, 765.

40. MYERS, B. A., AND SHEVKY, M. C.: The estimation of inorganic phosphorus in blood plasma by the method of Bell and Doisy. *J. Lab. Clin. Med.*, 1921–22, **7**, 176.

41. MYRBÄCK, E.: Zur Methodik der Calcium und Phosphorbestimmung in kleinen Blutmengen. *Z. physiol. Chem.*, 1925, **148**, 197.
42. NEUBAUER, H., AND LÜCKER, F.: Über die von Lorenzsche Methode der Phosphorsäurebestimmung. *Z. anal. Chem.*, 1912, **51**, 161.
43. NEUMANN, A.: Einfache Veraschungsmethode (Säuregemisch-Veraschung) und Vereinfachte Bestimmung von Eisen, Phosphorsäure, Salzsäure, und anderen Aschenbestandtheilen unter Benutzung dieser Säuregemische-Veraschung. *Z. physiol. Chem.*, 1902, **37**, 115.
44. PEMBERTON, H.: On the Determination of phosphoric acid. *J. Am. Chem. Soc.*, 1893, **15**, 382; 1894, **16**, 279.
45. PINCUS, Maasanalytische Bestimmung der Phosphorsaeure durch essigsaures Uranoxyd. *Virchow's Arch.*, 1859, **16**, 137.
46. POUGET, L., AND CHOUCHAK, D.; Dosage colorimétrique de l'acide phosphorique. *Bull. soc. chim. de Paris*, 1909, **5**, (4th series); 104; 1911, **9**, 649.
47. PREGL, F.: Die quantitative organische Microanalyse. 2nd. Ed. Berlin, 1923.
48. RANDLES, F. S., AND KNUDSON, A.: The estimation of lipoid phosphoric acid ("lecithin") in blood by application of the Bell and Doisy method for phosphorus. *J. Biol. Chem.*, 1922, **53**, 53.
49. ROCHE, J.: Recherches sur la précipitation du phosphore a l'état de phosphomolbydate de strychnine (Methode d'Embden). Applications au microdosage des diverses "formes" de phosphore du sang et du phosphore combiné dans les substances organiques sèches où en solution. *Bull. Soc. Chim. Biol.*, 1928, **10**, 1061.
50. ROE, J. H., IRISH, O. J., AND BOYD, J. I.: A study of the molybdic oxide colorimetric method for the estimation of the phosphorus compounds of the blood. *J. Biol. Chem.*, 1926, **67**, 579.
51. SOERENSEN, MARGRETHE: On the determination of small quantities of phosphorus in proteins. *Compt. rend. trav. lab. Carlsberg.*, 1923–25, **15**, No. 10.
52. TAYLOR, A. E., AND MILLER, C. W.: On the estimation of phosphorus in biological material. *J. Biol. Chem.*, 1914, **18**, 215.
53. TISDALL, F. F.: A rapid colorimetric method for the quantitative determination of the inorganic phosphorus in small amounts of serum. *J. Biol. Chem*, 1922, **50**, 329.
54. VAN SLYKE, D. D.: Unpublished Observations.
55. WHITEHORN, J. C.: A method for the determination of lipoid phosphorus in blood and in plasma. *J. Biol. Chem.*, 1924–25, **62**, 133.

CHAPTER XXXII

SULFUR

DISCUSSION

Methods used for sulfur in urine

In all standard methods for the analysis of urine, sulfur is determined as sulfate. The inorganic sulfates can be precipitated at once as barium sulfate, while neutral sulfur and ethereal sulfates must first be converted to inorganic sulfate by processes of oxidation and hydrolysis respectively. In the earlier methods sulfur was determined gravimetrically as barium sulfate.

In 1913 Gauvin and Skarzynski (8) and, in the next year, Rosenheim and Drummong (18) and Raiziss and Dubin (17) proposed the volumetric benzidine procedure. In this method sulfate is precipitated by benzidine hydrochloride as benzidine sulfate and is then titrated with alkali. Benzidine is a weak organic base which forms stable salts with strong mineral acids. The sulfate is insoluble, especially in the presence of excess benzidine and hydrochloric acid; its solubility is further diminished by the addition of acetone. When sodium hydroxide is added to a suspension of benzidine sulfate, the latter is decomposed according to the reaction:

$$C_{12}H_8(NH_2)_2 \cdot H_2SO_4 + 2\ NaOH = C_{12}H_8(NH_2)_2 + Na_2SO_4 + 2\ H_2O$$

The free benzidine is so weak a base that it is not alkaline to phenolphthalein or phenol red. Consequently the H_2SO_4 combined with it may be titrated as so much free acid.

The application of this method to urine is somewhat complicated by the simultaneous presence of phosphates and chlorides. Benzidine phosphate may be precipitated with the sulfate unless the urine is made strongly acid with HCl. But if enough acid is added to prevent phosphate precipitation, the urinary chlorides, if present in maximal concentration, may significantly increase the solubility of the benzidine sulfate. Fiske (5) in 1912 showed that the methods of Gauvin and Scharzinski, and of Rosenheim and Drummond, although satisfactory for analysis of most specimens of urine, failed when they were applied to specimens containing maximal amounts of chlorides or phosphates. To overcome the difficulty Fiske removed the phosphates. In their absence it is possible to avoid trouble from the chlorides by precipitating the benzidine sulfate at a lower acidity.

Quantitative methods are also available for the determination of certain specific sulfur compounds in urine. For cystine Looney (15) has provided a colorimetric procedure. Thiosulfates can be determined by iodometric titration, according to Holbøll (9) and Nyiri (16).

Methods used for non-protein sulfur in blood

The extreme dilution of inorganic sulfate, about 1 mg. of S per 100 cc. of blood, has rendered unusually difficult the problem of devising even moderately accurate methods for small amounts of blood.

In 1924 Klinke (13) proposed a micro iodometric titration. To the SO_4 solution was added a known volume of M/400 K_2CrO_4 followed by an equal volume of M/400 $BaCl_2$. In the absence of SO_4 both Ba and CrO_4 would be completely precipitated as $BaCrO_4$, but in the presence of SO_4 part of the Ba is precipitated as $BaSO_4$, leaving an equivalent of CrO_4 in solution, where it is titrated iodometrically. The results were not stoichiometric, but could be accurately interpreted by an empirical curve. The method was stated to be applicable to samples as small as 0.01 mg. of S, and therefore to filtrates from 1 cc. of blood. It does not appear to have been adopted by other authors, however, nor to have been applied to blood by Klinke himself.

Kahn and Postmontier (12) applied the method of Vansteenberghe and Bauzil (19), in which SO_4 is precipitated by excess $BaCl_2$, and the excess Ba is precipitated as $BaCO_3$, which is titrated with HCl. Loeb and Benedict (14) determined SO_4 gravimetrically in serum filtrate. Both of these methods were successfully applied to clinical material by their authors, but required 10 cc. samples of serum.

The methods which have been most extensively employed are those of Denis. In the first procedure (3) which she presented, blood proteins were removed with mercuric chloride and hydrochloric acid, a colloidal suspension of barium sulfate was produced in the filtrate by the addition of ammonium nitrate and barium chloride, and the quantity of sulfate was determined by means of the nephelometer. This procedure presents all the difficulties inherent in nephelometric techniques. It requires filtrate from 3 to 5 cc. of blood or plasma for an analysis. Denis and Reed (4) have proposed a method that permits the separate determination of the several sulfur fractions of blood: total sulfur, inorganic sulfate and ethereal sulfate. This is a modification of Denis's earlier nephelometric procedure with adaptions of the conventional methods for the different sulfur fractions.

The principle of benzidine precipitation of sulfate appears first to have been applied to the determination of sulfate in small amounts of blood by

Yoshimatsu (21). After precipitating the sulfate from a protein-free filtrate of blood with benzidine in alcoholic solution, he treated the benzidine sulfate, redissolved in water, with a mixture of iodine, potassium iodide and ammonia. The brown color that develops is compared in the colorimeter with that of a standard solution of benzidine sulfate treated in a similar manner. The technique is part of a general system for the colorimetric analysis of blood for inorganic elements which has been developed by Yoshimatsu.

Hubbard (10) and Kahn and Leiboff (11) have also determined benzidine sulfate precipitates colorimetrically. Hubbard utilized the yellow color produced by the action on benzidine of dilute hydrogen peroxide and ferric chloride. Kahn and Leiboff diazotized the benzidine sulfate and mixed it with phenol in an alkaline medium to produce a yellow color. Hubbard determined only inorganic sulfates. Wakefield (20) has presented certain modifications of the procedure which, he claims, render it suitable for the separate determination of the several sulfur fractions of blood or serum. An analysis can be done on filtrate equivalent to 1 cc. of blood or plasma.

The methods for the analysis of blood which have been chosen for presentation, are the gravimetric method of Loeb and Benedict (14), the nephelometric procedure of Denis and Reed (4), and Wakefield's (20) modification of Hubbard's (10) colorimetric method. The authors have had no personal experience with blood sulfur methods; therefore this selection is more or less arbitrary. For the analysis of urine and feces the titrimetric method of Fiske (5) and the gravimetric method of Folin and Benedict (1, 6, 7) are offered.

INORGANIC AND ETHEREAL SULFATES AND TOTAL SULFUR IN URINE. GRAVIMETRIC METHODS OF FOLIN AND BENEDICT (1, 6, 7)

Reagents

Dilute hydrochloric acid. One volume of concentrated hydrochloric acid diluted to 4 volumes.

A 5 per cent solution of barium chloride.

Benedict's total sulfur reagent. Dissolve 20 grams of copper nitrate crystals and 5 grams of potassium chlorate in water and dilute to 100 cc.

Procedures

***Inorganic sulfate* (*Folin*).** **In an Erlenmeyer flask place about 100 cc. of water, 10 cc. of dilute hydrochloric acid, and 25 cc. of urine. (If the urine is unusually dilute, take 50 cc. instead of 25 cc., and a corre-**

spondingly smaller amount of water.) Add 10 cc. of barium chloride solution, drop by drop. The urine must not be disturbed while the barium chloride is being added. At the end of an hour or later shake the mixture and filter it through a Gooch crucible, which has been weighed after ignition and cooling in room air. Wash the precipitate with at least 200 cc. of water. The crucible and its contents are then dried, ignited, cooled and weighed.

In igniting the barium sulfate precipitates the flame must not be applied directly to the bottom of the crucible or mechanical losses will occur. The Gooch crucible is dried at 100° to 120°, is then placed inside an ordinary porcelain or silica crucible, and the flame is applied to the latter, first gently, and finally with full force.

***Sum of inorganic and ethereal sulfates** (Folin).* In an Erlenmeyer flask into which a funnel has been placed to reduce the loss of steam, boil gently for twenty to thirty minutes 25 cc. of urine and 20 cc. of dilute hydrochloric acid (or 50 cc. of urine and 4 cc. of concentrated hydrochloric acid). This procedure hydrolyzes ethereal sulfate to free sulfuric acid. Cool the flask from two to three minutes in running water and dilute the contents with cold water to about 150 cc. The sulfate is then precipitated and weighed, as described above.

***Total sulfur** (Benedict).* To 10 cc. of urine in a porcelain evaporating dish of 7 or 8-cm. diameter add 5 cc. of Benedict's total sulfur reagent. Evaporate the contents of the dish over a free flame which is regulated to keep the solution just below the boiling point so that no loss will occur through spattering. When dryness is reached the flame is increased until the entire residue is blackened. The flame is then turned up in two stages to the full heat of the Bunsen burner and the contents of the dish are finally heated to redness for ten minutes after the black residue, which first fuses, has become dry. This final heating decomposes the last traces of nitrate and chlorate. The flame is then removed and the dish is allowed to cool. Ten to 20 cc. of the dilute hydrochloric acid are now added, and the dish is warmed gently until the contents have completely dissolved and a perfectly clear, sparkling solution results.

This solution is washed quantitatively into an Erlenmeyer flask and diluted to 100 or 150 cc. with cold water. The sulfate is then precipitated and weighed as described above.

Calculation

$0.1374\ W$ = grams of sulfate S in sample analyzed.

$\frac{137.4\ W}{V}$ = grams of sulfate sulfur calculated as S per liter of urine.

$$\frac{4286\,W}{V} \quad \text{millimoles of sulfate per liter of urine.}$$

$$\frac{8572\,W}{V} \quad \text{milli-equivalents of sulfate per liter of urine.}$$

W = weight of $BaSO_4$ in grams, V = cubic centimeters of urine taken for analysis,

$$\text{Total S} - (\text{ethereal} + \text{inorganic S}) = \text{Neutral S.}$$

$$(\text{Ethereal} + \text{inorganic S}) - \text{inorganic S} = \text{Ethereal S.}$$

INORGANIC AND ETHEREAL SULFATES AND TOTAL SULFUR IN URINE. FISKE'S BENZIDINE TITRATION METHOD (5)

The sulfate sulfur in the urine, before and after hydrolysis with acid, is precipitated with benzidine and the benzidine sulfate is determined by titration with alkali. Phosphate is first removed by precipitation as ammonium magnesium phosphate.

Reagents

Phenolphthalein indicator solution.

Strong ammonia water.

A 5 per cent ammonium chloride solution.

Powdered basic magnesium carbonate.

A 0.04 per cent alcoholic solution of brom-phenol blue.

Hydrochloric acid, approximately 1 N. (Dilute 80 cc. of concentrated hydrochloric acid (specific gravity 1.2) to 1 liter.)

Benzidine reagent. Suspend 4 grams of benzidine in about 150 cc. of water in a 250-cc. volumetric flask. Add 50 cc. of 1 N hydrochloric acid. Shake the flask until the benzidine is dissolved and then fill it to the mark with water. Filter the solution if necessary.

A 0.05 per cent aqueous solution of phenolsulfonephthalein (phenol red).

A 0.02 N sodium hydroxide solution.

An approximately 3 N solution of hydrochloric acid. Dilute 240 cc. of concentrated hydrochloric acid (specific gravity 1.2) to a liter.

Benedict's total sulfur reagent (1). Dissolve 20 grams of copper nitrate crystals and 5 gm. of potassium chlorate in water and dilute to 100 cc.

A 50 volume per cent solution of acetone.

A 95 volume per cent solution of acetone.

Procedure

Removal of phosphate. Dilute 15 cc. of urine (enough to contain 5 to 10 mg. of sulfur), in a 50-cc. volumetric flask, to about 25 cc. with water, and add a drop of phenolphthalein solution. Add concentrated ammonia drop by drop till the solution is faintly pink, and then introduce 5 cc. of a 5 per cent solution of ammonium chloride. Fill the flask to the mark with water, mix and pour the liquid into a dry Erlenmeyer flask containing about 0.65 gram of finely powdered basic magnesium carbonate. Shake the flask for one minute and transfer to a 9-cm. filter paper enough of the suspension to fill the filter nearly to the top. Allow this first filtrate to drain back into the Erlenmeyer flask; then filter the entire mixture through the same filter into a dry container. The phosphate-free filtrate thus prepared is used for the determination of all three kinds of sulfur.

Inorganic sulfate. To 5 cc. of the filtrate in a 200-cc. wide mouthed Erlenmeyer flask add 2 drops of a 0.04 per cent alcoholic solution of brom phenol blue and 5 cc. of water. Add, drop by drop, approximately 1.0 N hydrochloric acid till the blue color is dispelled and the solution is pure yellow. Run in, from a pipette, 2 cc. of benzidine reagent, and let the mixture stand two minutes.

Add 4 cc. of 95 per cent acetone and set the beaker aside ten minutes more. Filter through the special filtration tube, described on page 864. Wash the beaker and the filter with three 1-cc. portions and then with one 5-cc. portion of 95 per cent acetone.

In place of using Fiske's filter-tube and washing technique, one may collect and wash the precipitate by the technique described on p. 721 for washing the benzidine sulfate obtained in the total base determination.

Transfer the filter paper to the flask in which the precipitation was carried out, add 2 to 3 cc. of water and 2 drops of 0.05 per cent aqueous phenol red, and run in from a burette about 1 cc. of 0.02 N sodium hydroxide. Heat the mixture just to boiling and agitate the paper with the stirring rod. Wash down the sides of the flask with enough water to bring the volume to about 10 cc. and titrate with 0.02 N sodium hydroxide, stirring the paper around with the rod meanwhile. When the solution begins to turn red, heat it to boiling again. While it is still boiling complete the titration, adding not more than 0.02 cc. of the alkali at one time, until the solution acquires a definite pink color which is not discharged by further boiling. The purpose of the boiling is to accelerate resolution of the benzidine sulfate precipitate.

Sum of inorganic and etheral sulfates. To 5 cc. of the phosphate-free filtrate in a 200-cc. wide-mouthed Erlenmeyer flask add 1 cc. of approximately 3 N hydrochloric acid. Evaporate the solution to dryness on a boiling water bath and continue heating the dry material on the bath for ten minutes. Add 10 cc. of water immediately and break up the residue by rotating the flask. Add 2 cc. of the benzidine reagent and, two minutes later, 4 cc. of 95 per cent acetone. Then proceed with the sulfate analysis as described for inorganic sulfate.

Total sulfur. To 0.25 cc. of Benedict's total sulfur reagent in a 6-cm. evaporating dish, add 5 cc. of urine filtrate. Evaporate the mixture to dryness, preferably on an electric hot plate at low heat. When it is dry, increase the heat gradually to a maximum and finally ignite the material with a micro burner, continuing two minutes at red heat after the contents of the dish have become thoroughly black. Cool for five minutes. Add 1 cc. of 3 N hydrochloric acid and evaporate to dryness on the hot plate at low heat. When the residue is thoroughly dry, dissolve it and wash it into a 200-cc. wide-mouthed Erlenmeyer flask with five 2-cc. portions of water. Add 1 drop of 1.0 N hydrochloric acid, and precipitate with benzidine and acetone as in the determination of inorganic sulfate. The rest of the determination is also the same, except that, in order to wash the filter free from copper, a portion of 2 cc. of 50 per cent acetone must be used, instead of the first of the three 1-cc. portions of 95 per cent acetone.

Calculation

$$0.32\ (A - C) = \text{milligrams of S in sample.}$$

$$\frac{0.32\ (A - C)}{V} = \text{grams of S per liter of urine}$$

$$\frac{10\ (A - C)}{V} = \text{millimoles of sulfate per liter of urine.}$$

$$\frac{20\ (A - C)}{V} = \text{milli-equivalents of sulfate per liter of urine.}$$

A = cubic centimeters of 0.02 N alkali used in titration, C = correction for cubic centimeters of 0.02 N alkali used in the titration of a blank analysis on the reagents, V = cubic centimeters of urine in sample.

Neutral and ethereal sulfur are calculated as described for the Folin gravimetric method.

DETERMINATION OF SULFUR IN FECES

Sulfur is found in stools in the neutral form, as inorganic sulfate and also as ethereal sulfates. The methods described for the analysis of urine can be applied to the partition of the sulfates in stools. Certain modifications must be introduced, however. For the estimation of ***inorganic*** and ***ethereal sulfates*** ten grams of dried, pulverized feces (for preparation see p. 78), are extracted with water, and the extract is analyzed as described for urine.

For total sulfur the stool sample is suspended in water and ashed by the Benedict method described above.

INORGANIC SULFATE IN SERUM. GRAVIMETRIC DETERMINATION. LOEB AND BENEDICT (14)

The sulfate in filtrate representing 6 cc. of serum is precipitated and weighed in the classical way as $BaSO_4$. The weight of precipitate obtained from normal serum is only about 0.5 mg., and the error is probably greater than in the nephelometric and colorimetric methods. In nephritic retention, however, precipitates of several milligrams are obtained, and it is probable that the gravimetric method is the more accurate, as it permits weighing such small amounts, even with macro apparatus, to within 0.1 mg.

Reagents

Picric acid, saturated water solution.
Barium chloride, 1 per cent solution.

Procedure

One drop of caprylic alcohol and 10 cc. of serum are placed in a 50-cc. volumetric flask. Ten cubic centimeters of water and 30 cc. of saturated picric acid solution are added. After thorough mixing the contents are centrifuged rapidly, to avoid evaporation, and the supernatant fluid is filtered. A 30-cc. portion of the filtrate is placed in a 50-cc. beaker and 5 cc. of 1 percent barium chloride are slowly added. The solution is allowed to stand at least six hours for precipitation to become complete, and is then filtered through a 7-cm. ashless filter paper. Sometimes 2 to 4 filtrations are necessary before all the precipitate is retained on the paper. The latter is washed 5 times with 4 cc. portions of water acidulated with HCl. A small platinum crucible is ignited empty, cooled in the room air, and weighed. The filter paper is placed in the crucible and ignited slowly, the lid of the crucible being kept only slightly open

until charring has occurred. Later the lid is kept half open to prevent excessive reduction of $BaSO_4$. Care is taken not to allow the paper to burst into flame. Ignition should take about twenty-five minutes. The crucible is again cooled in room air and weighed at once.

Calculation

$0.1374\ W$ = milligrams of S in sample precipitated.

$\frac{13.74\ W}{V}$ = milligrams of S per 100 cc. of serum.

$\frac{4.29\ W}{V}$ = millimoles of sulfate per liter of serum.

$\frac{8.57\ W}{V}$ = milli-equivalents of sulfate per liter of serum.

W = milligrams of $BaSO_4$, V = cubic centimeters of serum represented in sample (in analyses performed as above directed, $V = 6$).

INORGANIC AND ETHEREAL SULFATES AND TOTAL NON-PROTEIN SULFUR IN BLOOD. NEPHELOMETRIC METHOD OF DENIS AND REED (4)

The blood proteins are removed by precipitation with 5 per cent trichloroacetic acid. The inorganic sulfate of the filtrate is determined directly by precipitating the sulfate in the filtrate by means of barium chloride, stabilizing the suspension with the aid of gelatin, and comparing the suspension in the nephelometer with standard sulfate suspensions which are prepared in a similar manner. Ethereal sulfates are determined in a similar manner in filtrates which have been previously subjected to acid hydrolysis; total sulfur in filtrates which have been treated with a zinc nitrate oxidizing mixture.

Reagents

Trichloroacetic acid. A 20 per cent solution prepared from sulfate-free trichloroacetic acid. Even high grade brands of the acid, as purchased, contain traces of sulfates. These can be readily removed by redistilling the acid *in vacuo* at a pressure of 15 to 20 mm. It is usually necessary to filter the solution through sulfate-free filter paper to remove suspended particles.

A 1.0 per cent barium chloride solution.

A 5.0 per cent barium chloride solution.

An 0.6 N sodium hydroxide solution. This should be prepared from metallic sodium. Sodium hydroxide as purchased appears to contain appreciable amounts of sulfate.

1.0 N hydrochloric acid.

0.1 N hydrochloric acid.

A 5 per cent solution of sulfate-free gelatin. As even high grade commercial gelatin contains traces of sulfates, it must be purified in the following manner. To 50 grams of high grade commercial gelatin in a flask, add 900 cc. of approximately 0.01 N hydrochloric acid and 100 cc. of a 5 per cent solution of barium chloride. Immerse the flask in a boiling water bath, shaking occasionally, for one hour. At the end of this period remove the flask from the boiling water, cool it in running water, add 50 cc. of egg-white, stir the mixture thoroughly, and heat it again in a boiling water bath for about thirty minutes or until the egg white has coagulated and carried down all traces of colloidal barium sulfate. Centrifuge the mixture, while it is hot, in 50 or 100-cc. tubes. Pour the clear supernatant liquid into small, wide mouthed bottles of about 30-cc. capacity. Stopper these with cotton, sterilize them by heating for an hour in boiling water, and after they have cooled store them in the refrigerator.

Zinc nitrate oxidizing mixture. Dissolve 25 grams of zinc nitrate, 25 grams of sodium chloride and 10 grams of ammonium chloride in 100 cc. of water. When the salts have been dissolved, filter the solution through ashless filter paper. The reagent must, of course, be tested for the presence of sulfate

Standard potassium sulfate solution. Two standards are required. The first contains 0.5437 gram of K_2SO_4 per liter. One cubic centimeter of this solution is equivalent to 0.1 mg. of S. The second standard is half as strong and is made by diluting 1 volume of the first standard with 1 volume of water.

Procedure

Precipitation of proteins of blood or serum. To 5 parts of citrated[1] blood or plasma are added 11 parts of water. After the solutions have been mixed, 4 parts of 20 per cent trichloroacetic acid are introduced. After the mixture has been thoroughly shaken it is allowed to stand for fifteen minutes and then centrifuged. The supernatant liquid is filtered through acid-washed dry filter paper, such as Whatman No. 40.

Inorganic sulfate of normal human blood. To 25 cc. of the

[1] Denis and Reed prefer citrate to oxalate as an anticoagulant because of the relative insolubility of barium oxalate.

protein-free filtrate add 4 cc. of 0.6 N sodium hydroxide, 1 cc. of 5 per cent gelatin and 1 cc. of 5 per cent barium chloride. After it has been mixed the suspension is allowed to stand fifteen minutes before it is read in the nephelometer against a standard solution that has been simultaneously prepared by adding to 1 cc. of the half strength potassium sulfate standard (containing 0.05 mg. of S per 1 cc.), 25 cc. of 0.1 N hydrochloric acid, 3 cc. of 0.6 N sodium hydroxide, 1 cc. of 5 per cent gelatin, and 1 cc. of 5 per cent barium chloride.

Inorganic sulfate of nephritic blood. For the analysis of blood from nephritic patients, which contains unusually large amounts of sulfate, a slightly different procedure is employed. Fifteen cubic centimeters of the protein-free filtrate treated with 2 cc. of 0.6 N sodium hydroxide, 1 cc. of 5 per cent gelatin and 5 cc. of 1 per cent barium chloride, are compared with 1 cc. of the stonger potassium sulfate standard (with 0.1 mg. S per 1 cc.) treated with 14 cc. of 0.1 N hydrochloric acid, 2 cc. of 0.6 N sodium hydroxide, 1 cc. of 5 per cent gelatin and 5 cc. of 1 per cent barium chloride.

Inorganic plus ethereal sulfate of normal human blood. To 15 cc. of protein-free filtrate in a 20 by 200-mm. Pyrex test tube add 4 cc. of 1.0 N hydrochloric acid and evaporate the solution at such a rate that solid particles begin to settle out in not less than fifteen to twenty minutes. At this point heating is discontinued. The evaporation of the last few cubic centimeters of liquid must be carried out with care to avoid undue discoloration. To the residue in the tube add 15 cc. of water, 2 cc. of 0.6 N sodium hydroxide, 1 cc. of 5 per cent gelatin and 5 cc. of 1 per cent barium chloride. After the solutions have been mixed the suspension is allowed to stand fifteen minutes before it is compared in the nephelometer against a standard which has been simultaneously prepared in the following manner. One cubic centimeter of potassium sulfate standard (it is well to prepare two standards: 0.05 mg. per 1 cc. and 0.1 mg. per 1 cc.) is evaporated, after addition of 4 cc. of 1.0 N hydrochloric acid, in the same manner as the blood filtrate. To the residue is added 15 cc. of water, 2 cc. of 0.6 N sodium hydroxide, 1 cc. of 5 per cent gelatin and 5 cc. of 1 per cent barium chloride.

Inorganic plus ethereal sulfate of nephritic blood. This is determined in the same manner as that of normal blood except that only 10 cc. of filtrate are used.

Total non-protein sulfur of normal human blood. To 5 or 10 cc. of blood filtrate add 1 cc. of zinc nitrate reagent. Evaporate the mixture to dryness in a 20 by 200-mm. Pyrex test tube, and continue to heat

it with a free flame until fumes are no longer given off. Dissolve the residue in 2 cc. of 1.0 N hydrochloric acid, add 15 cc. of water, 1 cc. of 5 per cent gelatin and 5 cc. of 1 per cent barium chloride. After fifteen minutes compare the suspension in the nephelometer against a standard prepared at the same time as the unknown in the following manner. One cubic centimeter of standard potassium sulfate (it is well to prepare 2 standards: 0.1 mg. of S per cubic centimeter and 0.05 mg. of S per cubic centimeter) and 1 cc. of oxidizing reagent are evaporated to dryness and heated as described above. The residue, dissolved in 2 cc. of 1.0 N hydrochloric acid, is treated with 15 cc. of water, 1 cc. of 5 per cent gelatin and 5 cc. of 1 per cent barium chloride.

Total non-protein sulfur of nephritic blood. This is determined in the same manner as that of normal blood except that only 3 to 5 cc. of blood filtrates are used.

Calculation

$$\frac{400\,DS}{U\,V} \quad \text{milligrams of S per 100 cc. of blood.}$$

$$\frac{125\,DS}{U\,V} = \text{millimoles of sulfate per liter of blood.}$$

$$\frac{250\,DS}{U\,V} = \text{milli-equivalents of sulfate per liter of blood.}$$

S and U represent nephelometer readings of standard and unknown respectively, V, the number of cubic centimeters of filtrate taken for an analysis and D, the milligrams of S in the 1 cc. of standard solution used.

The neutral and ethereal sulfur are calculated as described for the Folin urine analysis.

Precautions

The usual precautions in the use of the nephelometer must be observed. Satisfactory colloidal precipitation will not occur if the amount of sulfur in 25 cc. volume exceeds 0.16 to 0.2 mg. or is less than 0.02 mg. Since the size of the particles in suspension depends to some extent on the amount of sulfate present, standard and unknown must be of approximately the same strength. It is therefore, well to prepare two standards for each determination.

If the reaction of the mixture varies greatly from a pH of 3.0 to 3.8 barium sulfate precipitation is not quantitative. If the mixture is too alkaline

barium phosphate is precipitated; if it is too acid the soluble acid barium sulfate is formed. Trichloroacetic acid filtrates of blood from pathological cases are sometimes more acid than usual, presumably because the blood is deficient in protein. In this case the amount of sodium hydroxide may have to be varied. If, upon precipitation of a filtrate, no cloud appears, it is advisable to determine the pH of the filtrate and adjust this to the proper reaction.

If the oxidation mixture used in the determination of total sulfur is not heated until all the nitrate is decomposed and driven off, very low results are obtained.

INORGANIC AND ETHEREAL SULFATES IN BLOOD. HUBBARD'S (10) COLORIMETRIC METHOD AS MODIFIED BY WAKEFIELD (20)[2]

Sulfate is precipitated by means of benzidine: the precipitate is treated with dilute hydrogen peroxide and ferric chloride, and the resultant yellow color is compared with that of a standard solution which has been treated in a similar manner.

Reagents

0.2 N hydrochloric acid.

Benzidine standards. 2.007 grams of benzidine hydrochloride are dissolved in 500 cc. of 0.2 N hydrochloric acid. One cubic centimeter of this solution is equivalent to 1.5 mg. of SO_4, or 0.5 mg. of S. From this solution, by 10, 50, and 100-fold dilution, working standards are prepared which contain amounts of benzidine equivalent to 0.15, 0.03 and 0.015 mg. of SO_4, or 0.05, 0.01, and 0.005 mg. of S per cubic centimeter. 0.2 N hydrochloric acid must be used as the diluent in every case.

Trichloroacetic acid, 20 per cent solution, free from sulfate (see method of Denis and Reed for preparation).

Hydrogen peroxide. One volume of commercial hydrogen peroxide diluted to 5 volumes with water.

Ferric chloride. A 1 per cent aqueous solution of ferric chloride, which must be freshly prepared each week.

Pure acetone. This must be redistilled, if necessary to insure purity.

Benzidine base. A 1 per cent solution of pure benzidine (Eastman organic or Merck's blue label is satisfactory) in pure acetone. The solution must be discarded as soon as a yellow color develops.

[2] Certain modifications of Wakefield's published procedure have been made at the suggestion of Roger S. Hubbard, who kindly corrected the manuscript of this section.

All reagents must, of course, be carefully tested for sulfate. Glassware must be scrupulously clean and free from sulfate. It may be cleaned in the ordinary potassium dichromate sulfuric acid cleaning solution; but all traces of the cleaning solution must be rinsed off with large amounts of water. All water which is used must be free from sulfate.

Procedure

Serum is collected by defibrination or spontaneous clotting of blood. (Citrated plasma may serve, but oxalated plasma or blood can not be used.)

Removal of serum proteins. To 3 cc. of the serum in a 15 cc. centrifuge tube add an equal volume of water, followed by 3 cc. of 20 per cent trichloroacetic acid. When these solutions have been mixed add enough water to bring the total volume to 15 cc. The contents of the tube are thoroughly mixed again and centrifuged for five minutes.

Determination of inorganic sulfate. Five cubic centimeters of the supernatant liquid are transferred to a second tube, containing 10 cc. of the acetone solution of benzidine base. The tube is capped, placed in ice water for an hour, or until the precipitate flocculates, and is then centrifuged for 30 minutes. The tubes must be capped to prevent evaporation of the acetone before and during centrifuging. The supernatant fluid is poured off and the tube is inverted for three minutes on a dry filter paper. The mouth of the tube is dried and 15 cc. of pure acetone are added; the precipitate, broken up with a fine point glass rod, is stirred in the acetone. The tube is again capped and centrifuged for fifteen minutes at 3000 revolutions per minute. The acetone is poured off and the tube inverted to drain on a filter paper, this time for five minutes. The lip of the tube is wiped with dry filter paper and 2 cc. of 0.2 N hydrochloric acid are added. If the precipitate is not dissolved by shaking, the tube may be warmed gently over a burner, but the contents must not be allowed to boil. If the tube has been heated it must be cooled or allowed to cool before proceeding. Six cubic centimeters of water are added, followed by 1 cc. of dilute hydrogen peroxide and 1 cc. of ferric chloride solution. After the contents of the tube have been thoroughly mixed they are set aside for five minutes to permit the color to develop.

Standards should be treated with hydrogen peroxide and ferric chloride at the same time as the unknown. The acidity of standard and unknown must be the same. For example, to 1 cc. of standard made in 0.2 N HCl, 1 cc. of 0.2 N hydrochloric acid must be added before

the water, peroxide and ferric chloride, because 2 cc. of 0.2 N hydrochloric acid were added to the unknown.

Standard and unknown should be compared in the colorimeter during the second five minutes after the peroxide and ferric chloride have been added. If readings can not be made within this interval 0.5 cc. of concentrated hydrochloric acid may be added to both standards and unknown to prevent the colors from fading.

Inorganic plus ethereal sulfate. The procedure for determination of total sulfate of serum is the same as that for inorganic sulfate except that the filtrate is hydrolyzed with hydrochloric acid. Two cubic centimeters of concentrated hydrochloric acid are added to 5 cc. of the protein-free filtrate in a centrifuge tube. The tube is placed in a beaker of boiling water for fifteen minutes. At the end of this time the tube is allowed to cool, or is cooled under running water, before the benzidine base solution is added. When the benzidine base solution is added a dense turbidity, due to the acetone and hydrochloric acid, forms; but this disappears as soon as the contents of the tube are thoroughly mixed. From this point on the procedure is the same as that for the determination of inorganic sulfate.

Calculation

$$\frac{100\ DS}{U} = \text{milligrams of } SO_4 \text{ in 100 cc. of serum.}$$

$$\frac{33.4\ DS}{U} = \text{milligrams of sulfate S in 100 cc. of serum.}$$

$$\frac{10.4\ DS}{U} = \text{millimoles of sulfate per liter of serum.}$$

$$\frac{20.8\ DS}{U} = \text{milli-equivalents of sulfate per liter of serum.}$$

S and U = colorimeter readings of standard and unknown respectively; D = milligrams of SO_4 to which the standard used is equivalent.

Precautions

It is important to select centrifuge tubes with fine pointed conical bottoms, because if the bottoms are too rounded precipitate may be lost during the process of washing.

When both sulfate and phosphate are present in high concentration (e.g., in terminal nephritis), phosphate may be precipitated with sulfate by

benzidine. If, therefore, the blood is known or suspected to contain high sulfate and high phosphate it is well to take less than the usual quantity for analysis. If an unusually bulky benzidine precipitate is obtained, such as may be contaminated by phosphate, the precipitate can be purified by redissolving it in 2 cc. of 0.2 N HCl. The acid is neutralized with 2 cc. of 0.2 N NaOH (sulfate-free), and the analysis is continued as described under the heading "Determination of inorganic sulfate." To the solution of dissolved precipitate add 5 cc. of 20 per cent trichloroacetic acid and dilute to 15 cc. with water. With a 5-cc. aliquot repeat the precipitation with benzidine and continue the determination as described. The value obtained must be multiplied by 3 to give the amount of S in the volume of serum originally taken for analysis.

MICRO TITRATION METHOD FOR BLOOD SULFATES

Cope has recently shown that, instead of determining the benzidine precipitates obtained in Hubbard's method by colorimetry, they can be satisfactorily titrated with 0.02 N sodium hydroxide with Rehberg's micro pipette. The method will be found in the appendix.

BIBLIOGRAPHY

1. Benedict, S. R.: The estimation of total sulfur in urine. *J. Biol. Chem.*, 1909, **6**, 363.
2. De Boer, S.: The influence of the respiration on the exchange of SO_4 between corpuscles and plasma and its effect on the excretion of SO_4. *J. Physiol.*, 1917, **51**, 211.
3. Denis, W.: Sulfates in blood. *J. Biol. Chem.*, 1921, **49**, 311.
4. Denis, W., and Reed, L.: Methods for the determination of some of the non-protein sulfur compounds of blood. *J. Biol. Chem.*, 1926–27, **71**, 191.
5. Fiske, C. H.: The determination of inorganic sulfate, total sulfate and total sulfur in urine by the benzidine method. *J. Biol. Chem.*, 1921, **47**, 59.
6. Folin, O.: Approximately complete analyses of thirty "normal" urines. *Am. J. Physiol.*, 1905, **13**, 45.
7. Folin, O.: On sulfate and sulfur determinations. *J. Biol. Chem.*, 1905–06, **1**, 131.
8. Gauvin, R., and Skarzynski, V.: Dosage rapide du soufre sous ses différents états dans les liquides biologiques et en particulier dans l'urine. *Bull. Soc. chim.*, 1913, Series 4, **13**, 1121.
9. Hølboll, S. A.: Untersuchungen über die Quantitative Bestimmung des Natriumthiosulfats im Urin mit besonderem Bezug auf die Anwendung des Stoffes als Nierenfunktionsprobe. *Klin. Woch.*, 1925, **4**, 1636.
10. Hubbard, R. S.: A colorimetric method for the determination of sulfate in serum. *J. Biol. Chem.*, (Proceedings), 1927, **74**, p. v.
11. Kahn, B. S., and Leiboff, S. L.: Colorimetric determination of inorganic sulfate in small amounts of urine. *J. Biol. Chem.*, 1928, **80**, 623.

12. Kahn, M., and Postmontier, R. S.: On the non-protein sulphur of the blood. *J. Lab. Clin. Med.*, 1925, **10**, 317.

13. Klinke, K.: Versuch einer massanalytischen Bestimmung des SO_4 Ions in kleinsten Mengen. *Biochem. Z.*, 1924, **154**, 171.

14. Loeb, R. F., and Benedict, E. M.: Inorganic sulphates in human blood. *J. Clin. Invest.*, 1927, **4**, 33.

15. Looney, J. M.: The colorimetric estimation of cystine in urine. *J. Biol. Chem.*, 1922, **54**, 171.

16. Nyiri, W.: Über die Thiosulfatprobe, eine neue Methode der Nierenfunktionsprüfung. *Klin. Woch.*, 1923, **2**, 204.

17. Raiziss, G. W., and Dubin, H.: A volumetric method for the estimation of total sulfur in urine. *J. Biol. Chem.*, 1914, **18**, 297.

18. Rosenheim, O., and Drummond, J. C.: A volumetric method for the estimation of ethereal and inorganic sulphates in urine. *Biochem. J.*, 1914–15, **8**, 143.

19. Vansteenberghe, P., and Bauzil, L.: Cited by Kahn and Postmontier (12), Volumetric determination of sulfates. *Ann. chim. anal.*, 1918, **23**, 210. *C. A.*, 1919, **13**, 460.

20. Wakefield, E. G.: The colorimetric determination of total and inorganic sulfates in blood serum, urine, and other body fluids. *J. Biol. Chem.*, 1929, **81**, 713.

21. Yoshimatsu, S.-I.: Colorimetric method for the determination of inorganic sulphates, inorganic phosphates and chlorides in small amounts of blood. *Tohoku J. Exp. Med.*, 1926, **7**, 553.

APPENDIX

THE PHENOLSULFONEPHTHALEIN TEST FOR RENAL FUNCTION

In 1909 Abel and Rowntree (1) in an investigation of the pharmacological action of a variety of phthalein derivatives, discovered that phenolsulfonephthalein was excreted almost entirely by the kidneys. The dye was quite free from toxic properties and, in the red alkaline form, lent itself to colorimetric determination. As a result of this investigation Rowntree and Geraghty (4) devised their clinical test, which consists of injecting a known amount of the dye and then determining colorimetrically the quantity excreted in the urine in a given interval. The procedure will be described as it was originally proposed, and the various modifications will be discussed subsequently.

Procedure

Preparation of the phenolsulfonephthalein solution. The solution is prepared by adding to 600 mg. of phenolsulfonephthalein 0.84 cc. of 2.0 N sodium hydroxide solution. The dye is dissolved with the aid of 0.75 per cent sodium chloride solution. A further 0.15 cc. of the 2.0 N sodium hydroxide is then added and the solution, transferred to a 100-cc. volumetric flask, is diluted to the mark with 0.75 per cent sodium chloride solution. The alkaline sodium salt of the dye is non-irritating. The solution can be sealed in hard glass ampoules, each containing slightly more than 1 cc. of solution, and sterilized.[1]

Preparation of the subject. Immediately before the test the patient is directed to drink 200 to 400 cc. of water to insure an adequate urine flow. A catheter is then inserted with the usual sterile precautions and the urine already in the bladder is withdrawn. Exactly 1 cc. of the dye solution is injected by means of a well ground syringe subcutaneously, intramuscularly or intravenously. The time of injection is carefully noted.

Determination of the appearance time. Immediately after the injection the urine is collected in a clean receptable containing a few drops of 25 per cent sodium hydroxide solution. Phenolsulfonephthalein, in acid solution, such as normal urine, has a golden yellow color that

[1] Ampoules of sterile phenolsulfonephthalein solution, standards and comparator can be secured from Hynson, Westcott and Dunning, Baltimore, Md.

is not easily distinguishable; the alkaline salt is a deep red. As soon as the dye appears in the urine the red color will appear on contact with the strong alkali. At this point the time is again noted and the catheter may be withdrawn unless there is known to be some obstructive condition of the urinary tract that prevents complete emptying of the bladder.

Total phthalein excretion. At the end of exactly one hour from the time of appearance of the dye the patient empties his bladder completely into the same receptable which was used to collect the catheterized sample. At the end of the second hour after the appearance of the dye the bladder is again emptied into a separate receptable.

Determination of the amount of phthalein excreted. To each of the two specimens 25 per cent sodium hydroxide is added slowly, with stirring, until the red color of the alkaline phthalein has reached its full intensity. The specimens are then diluted to suitable volumes and compared colorimetrically with known standard solutions of alkaline phenolsulfonephthalein.

Comparisons can be made in any of the usual types of colorimeters. In this case the standard is made by diluting 6 mg. of phenolsulfonephthalein to 1 liter with water which has been alkalinized with sodium hydroxide. The urine may be diluted to 500 or 1000 cc., depending upon the amount of dye which it contains. If the phthalein excretion is extremely small it is occasionally advantageous to dilute to a smaller volume unless the urine itself is too highly colored.

The results are recorded in terms of the per cent of injected dye which is recovered in the urine. This is calculated by means of the formula:

$$\frac{S\,V}{U\,1000} = \text{per cent of dye.}$$

Where S and U = the colorimetric readings of standard and unknown, respectively; V = the volume in cubic centimeters to which the unknown was diluted before the colorimetric comparison.

For practical clinical purposes comparisons may be made with a series of permanent standards in a simple comparator. These standards are prepared by dilution from a 100 per cent standard (6 mg. of alkaline phenolsulfonephthalein diluted to 1 liter) at 5 or 10 per cent intervals from 0 to 100 per cent. They can be sealed in test tubes of even bore. The unknown, properly diluted and alkalinized in a similar tube, is compared with these standards.[1]

If the urine contains an appreciable amount of hemolyzed blood the test cannot be carried out. If the specimens are not clear they may be centrifu-

gated. Numerous attempts have been made to eliminate the interference of blood and urinary pigments by the use of adsorbents. Most of these measures, however, remove, with the interfering substances, a certain amount of the dye.

The alkaline standard, if not sealed and sterilized, develops molds and gradually fades. It must, therefore, be renewed at frequent intervals.

Variations of the original technic

Except in genito-urinary surgery the appearance time is seldom determined. Catheterization is employed only when there is reason to suspect partial obstruction of the lower urinary tract. Urine specimens are usually collected 70 and 130 minutes after injection of the dye on the assumption that the dye appears in the urine within ten minutes. In point of fact there is no great value in prolonging the test beyond 130 minutes, because the dye which is not excreted within this period of time is usually destroyed in the body.

At first considerable importance was ascribed to differences in the quantities of dye excreted in the first and in the second hours. More extensive application has shown that such differences have no great significance. It has, therefore, become the custom in many clinics to collect only a single specimen, two hours and ten minutes after the injection of the dye.

Comparison of the function of the two kidneys

In urological practice the phenolsulfonephthalein test has proved extremely useful for the determination of the function of the two kidneys separately.

The patient is given 200 to 400 cc. of water. After this the ureters are catheterized through the cystoscope. Then 1 cc. (6 mg.) of phenolsulfonephthalein is injected intravenously and the time recorded. Urine from the catheters is collected in two separate receptables for twenty to thirty minutes. The appearance time and the total amount of dye excreted by each kidney are determined by the methods described above.

Mode of injection: Under ordinary circumstances it makes little difference in the two-hour excretion whether the dye is injected subcutaneously or intramuscularly. After intravenous administration the appearance time is shortened and elimination is more complete. In the presence of subcu-

taneous edema the absorption of phthalein from the subcutaneous tissues into the circulation seems to be delayed; appearance time is prolonged and the total excretion diminished. *It is, therefore, preferable to administer the dye intravenously, especially* to edematous patients.

It is essential that the bladder be emptied completely at the end of the test. In case of doubt it is well to collect another specimen of urine after the third hour. If this contains more than a trace of dye it is probable that the patient did not empty his bladder completely, possibly because there is some obstruction in the lower urinary tract.

Normal excretion. In normal subjects the dye should appear in the urine within 10 minutes after injection and at least 50 per cent, usually more, should be excreted within two hours and ten minutes. The greater portion of this appears in the first hour.

Pathological excretion. The relationship between renal disease and retarded phthalein excretion is discussed in the urea chapter of Volume I. The effects of other pathological conditions on phthalein excretion have been studied by Lundsgaard and Møller (2, 3), who have found the excretion retarded in a large proportion of cases of liver disease and of heart disease, compensated and uncompensated.

BIBLIOGRAPHY

1. Abel, J. J., and Rowntree, L. G.: On the pharmacological action of some phthaleins and their derivatives, with especial reference to their behavior as purgatives. I. *J. Pharm. Exp. Therap.*, 1909–10, **1**, 231.
2. Lundsgaard, C., and Møller, E.: Investigations into the value of the phenolsulfonephthalein test in renal and circulatory diseases. *Act. Med. Scand.*, 1925–6, **63**, 242.
3. Møller, E., and Lundsgaard, C.: Continued investigations into the excretion of phenolsulfonephthalein by the kidneys: the course of the excretion curve and the total excretion in circulatory and liver diseases. *Act. Med. Scand.*, 1925–26, **63**, 268.

   An investigation into the fate of phenolsulfonephthalein in the organism. *Ibid.*, 1925–26, **63**, 277.
4. Rowntree, L. G., and Geraghty, J. T.: The phthalein test. An experimental and clinical study of phenolsulphonephthalein in relation to renal function in health and disease. *Arch. Int. Med.*, 1912, **9**, 284.

THE BROMSULPHALEIN TEST OF LIVER FUNCTION

In 1909, in an investigation of the pharmacological action of some phthalein derivatives, Abel and Rowntree (1) found that phenol tetrachlorophthalein, when given subcutaneously, was excreted almost entirely in the bile. This observation was first applied to the study of liver function in

1913 by Rowntree, Bloomfield, and Hurwitz (5). They determined the amount of injected dye which was excreted in the stools. A similar procedure was reported in the same year by Whipple, Mason, and Peightal (6). Because of the difficulties and uncertainties of the technique McNeil (2) in 1916 suggested that bile collected by duodenal tube be analyzed, instead of feces. Neither method proved entirely practical or accurate because of the uncertainty of quantitative collection. In 1922 Rosenthal (3) demonstrated that if the dye was injected into a vein the greater part of it remained in the circulation until it was removed by the liver for excretion. He decided that it would be more simple and accurate to determine the rate at which the dye was removed from the blood than to attempt to measure directly the amount excreted. He therefore proposed the colorimetric determination of the amount of dye remaining in the blood serum at given intervals after intravenous injection. The method proved both practicable and useful. Subsequently Rosenthal and White (4) showed that bromsulphalein (phenoltetrabromphthalein sodium sulfonate) was excreted in the bile more rapidly and completely than phenoltetrachlorphthalein and lent itself better to colorimetric determination.

Reagents (4)

A sterile 5 per cent solution of bromsulphalein.

A 10 per cent solution of sodium hydroxide.

Standard solutions of bromsulphalein. Four milligrams of bromsulphalein are diluted to 100 cc. with 0.0005 *N* solution of sodium hydroxide. This solution is arbitrarily termed the 100 per cent standard. From it are prepared, by dilution with 0.0005 *N* sodium hydroxide solution, a series of standards at 5 or 10 per cent intervals from 0 to 100 per cent. The standards, sealed in hard glass test tubes of uniform bore, can be kept in the dark for several months without deterioration.[2]

Procedure

Injection of dye. **The test should be made before breakfast, when the subject is in the post absorptive state, to avoid alimentary lipemia. Two milligrams of dye per kilo of body weight (1/25 cc. of the 5 per cent solution per kilo or 1/55 cc. per pound) are injected intravenously by means of a well ground hypodermic syringe. It is essential that none of**

[2] Ampoules containing sterile bromsulphalein, standards in sealed tubes, and a convenient comparator box may be secured from Hynson, Westcott and Dunning, Baltimore, Md.

the dye be allowed to escape into the subcutaneous tissues. It is inadvisable to introduce the bromsulphalein too rapidly; the injection should take about sixty seconds.

Determination of dye retention. At the end of exactly five minutes after the injection and again after thirty minutes, venous blood is withdrawn, allowed to clot and then centrifuged. Care must be taken to avoid hemolysis. The serum is carefully withdrawn from each sample

To part of each sample of serum, in a tube similar to those containing the standard solutions, 2 drops of 10 per cent sodium hydroxide are added, to bring out the alkaline color of the dye. The remainder of the serum, placed in a third tube, is acidified with 1 drop of 5 per cent hydrochloric acid (5 cc. of concentrated hydrochloric acid diluted to 100 cc.) This acidified sample serves as a control in the color comparison. The unknown samples are compared with the standards in the usual manner in a comparator. The acidified serum is placed behind the standard, a tube containing water behind the unknown.

In subjects without liver disease the serum at the end of five minutes should give a reading of 20 to 50 per cent; after thirty minutes it should contain only a trace—less than 0.5 per cent—of dye.

BIBLIOGRAPHY

1. Abel, J. J., and Rowntree, L. G.: On the pharmacological action of some phthaleins and their derivatives, with especial reference to their behavior as purgatives. I. *J. Pharm. Exp. Therap.*, 1909–10, **1**, 231.
2. McNeil, H. L.: The quantitative estimation of phenoltetrachlorophthalein excreted in the fresh bile in disease of the liver. *J. Lab. Clin. Med.*, 1915–16, **1**, 822.
3. Rosenthal, S. M.: An improved method for using phenoltetrachlorophthalein as a liver function test. *J. Pharm. Exp. Therap.*, 1922, **19**, 385.
   A new method of testing liver function with phenoltetrachlorophthalein. *Johns Hopkins Hosp. Bull.*, 1922, **23**, 432.
4. Rosenthal, S. M., and White, E. C.: Studies in hepatic function. VI. A. The pharmacological behavior or certain phthalein dyes. B. The value of selected phthalein compounds in the estimation of hepatic function. *J. Pharm. Exp. Therap.*, 1924, **24**, 265.
   Clinical application of the bromsulphalein test for hepatic function. *J. Am. Med. Ass.*, 1925, **84**, 1112.
5. Rowntree, L. G., Hurwitz, S. H., and Bloomfield, A. L.: An experimental and clinical study of the value of phenoltetrachlorophthalein as a test for hepatic function. *Johns Hopkins Hosp. Bull.*, 1913, **24**, 327.
6. Whipple, G. H., Mason, V. R., and Peightal, T. C.: Tests for hepatic function and disease under experimental conditions. *Johns Hopkins Hosp., Bull.*, 1913, **24**, 207.

METHODS FOR THE DETERMINATION OF BILIRUBIN IN SERUM: THE ICTERUS INDEX; THE VAN DEN BERGH REACTION

As a measure of the rate of production of bile pigments and their excretion by the liver, and for the objective estimation of the nature and intensity of clinical jaundice, it has long been recognized that examinations of the excreta are inadequate. Various methods have, therefore, been proposed for the quantitative estimation of bilirubin in serum.

The simplest of these, the icterus index test, was introduced by Meulengracht (14). It consists of nothing more than the comparison, by dilution colorimetry, of the serum of the subject with a standard solution of potassium bichromate. The method has received wide application and proved extremely useful. It has, however, little to recommend it theoretically, except its simplicity. Meulengracht, when he proposed the method, admitted that its validity depended upon the assumption that there were no significant amounts of pigments other than bilirubin in serum. He recognized that this assumption was not entirely justified, that even normal serum contained traces of lipochrome pigments; but he believed that even in disease the concentrations of such pigments in the serum were negligible. Since then it has been repeatedly demonstrated that this is not the case. In carotinemia and in diabetes lipochromo pigments may accumulate in the serum in quantities large enough to give icterus indices as high as those found in obstructive jaundice (3, 7). In spite of this source of error the method, intelligently interpreted, is of distinct value in the diagnosis of diseases of the liver, bile ducts, and blood-forming organs.

The method of Hymans van den Bergh is, however, theoretically sounder and of broader clinical significance. Van den Bergh and Snapper (2), in 1913, showed that Ehrlich's diazo reaction for bilirubin could be applied to serum. This reaction, first discovered by Ehrlich (4, 5) in 1883 and later studied by Pröscher (16), depends on the fact that bilirubin, in alcoholic solution, reacts with a diazonium salt to form an acid dye, azo-bilirubin. Van den Bergh found that the dye lent itself to colorimetric determination in serum. Lipochromes and hemoglobin derivatives do not appear to affect the color (6, 9, 18). The above react is called the *indirect.*

Subsequently Van den Bergh and Muller (1) discovered that under certain circumstances a *blue* color developed promptly when the diazo reagent was added to serum without alcohol. This reaction, known as the *prompt direct reaction,* occurs only in cases of obstructive jaundice. In hemolytic jaundice, on the other hand, either no color appears when the diazo reagent is added directly to the serum, or else a *red* color develops only gradually

after a latent period of a minute or more and does not reach the intensity attained in the prompt direct reaction. This is known as the *delayed direct reaction*. Feigl and Querner (6) later reported a third type of reaction which they called the *biphasic reaction*. In this a reddish color appears immediately after the diazo reagent is added to the serum, later rapidly or gradually changing to violet.

The application of both direct and indirect van den Bergh tests to serum not only more or less closely shows the quantity of bilirubin in serum, but also gives information of value concerning the pathogenis of the bilirubinemia. Regardless of the nature of the direct reaction, the indirect—i.e., the development of color in the alcoholic extract of serum—is always positive and affords a measure of the concentration of bilirubin in the serum.

Concerning the causes of the variations in the direct reaction there has been considerable speculation. Van den Bergh and Muller (1) concluded that they were due either to: 1) differences in the nature of bilirubin or 2) combination of bilirubin with certain components of serum. The first hypothesis found many supporters who argued that, although bilirubin was formed outside the liver, it was subjected to chemical alteration by the liver cells. It was supposed that only bilirubin which had been transformed by the liver gave the direct reaction. This theory found its greatest support in the fact that bile secured from the gall bladder or biliary passages always gave a prompt direct reaction. The work of Mann and his associates (11) on the effects of total extirpation of the liver, besides finally disposing of all doubts concerning the extrahepatic origin of bilirubin, afforded an opportunity for the examination of extrahepatic bile pigments. No differences could be demonstrated between the bilirubin which appeared in the serum of hepatectomized animals and that obtained from bile.

Harrop and Barron (8) have attempted to analyze the nature of the direct reaction by *in vitro* experiments. They found that when pure bilirubin was added to normal human serum only a delayed reaction could be obtained until the concentration of bilirubin exceeded a certain level. After this the direct reaction appeared. Serum to which bile salts had been added gave a direct reaction with extremely low bilirubin concentrations. Other substances which reduced surface tension had an effect similar to that of bile salts. Harrop and Barron concluded that bile pigments are ordinarily adsorbed by certain constituents of serum, probably proteins; but are freed from such adsorption by bile salts and other surface tension-reducing substances. Only free pigments give a direct van den Bergh reaction. This theory affords an explanation not only of the variations in the direct van

den Bergh reaction; but also of the fact that bilirubin as such does not appear in the urine of patients with jaundice of extrahepatic origin. It is generally stated that the renal threshold for bilirubin is higher in these cases. It is far easier to believe that the bilirubin, by reason of the fact that it is held by the proteins, is not available for excretion by the kidneys. In contradiction to the conclusions of Harrop and Barron stand some earlier experiments of Thannhauser and Anderson (18). They were unable, by the addition of bile acids and cholesterol to the serum of a patient with hemolytic icterus, to convert a delayed to a prompt direct reaction. In fact they found that the addition of bile and cholesterol to the serum of subjects with obstructive jaundice abolished the direct reaction entirely.

THE DETERMINATION OF BILIRUBIN IN SERUM

Blood for both icterus index and van den Bergh tests should be taken while the patient is in the post-absorptive state. This avoids the clouding effect of alimentary lipemia and, in the case of the icterus index, of the recent ingestion of foods containing lipochrome. Bernheim (1) detected temporary increases in the icterus index of normal subjects after the ingestion of as little as one or two carrots. Such increases, however, disappear after the over night fast. The same blood sample may be used for both icterus index and van den Bergh. Usually 10 cc. suffices for both tests.

The blood, after it has been allowed to clot, is centrifuged and the serum separated with every precaution against hemolysis. The van den Bergh test must be carried out within 2 hours of time the blood is collected. If the serum is allowed to stand the reactions become altered. Sera which, when fresh, give direct reactions, may, after standing, give only the indirect reaction (13, 18).

Icterus Index

The only solution required is an 0.01 per cent solution of potassium bichromate, which serves as a standard. The serum is compared directly with the standard in a colorimeter. If the serum is too dark to permit color comparison it is diluted to the proper point with 0.9 per cent sodium chloride solution.

Calculation

The test is reported in terms of the relative color of serum and standard, the latter having the value of 1. Thus, if the color of the serum is 10 times

as intense as that of the standard, the index is said to be 10. The index can be calculated by the formula

$$\frac{S\,D}{U} = \text{icterus index},$$

S and U = readings of standard and unknown respectively, D = the number of times the unknown was diluted.

The icterus index of normal persons under standard conditions seldom exceeds 6. If it exceeds 15, clinical jaundice is usually demonstrable. However, values as high as this have been reported in patients with carotinemia who had no excess of bilirubin in their sera. Greene, Snell and Walters (7) in one such case found an icterus index of 26.

van den Bergh test

Reagents. Ehrlich's diazo reagent. A. One gram of sulfanilic acid and 15 cc. of concentrated hydrochloric acid diluted to 1 liter.

B. 0.5 gram of sodium nitrite diluted to 100 cc.

These solutions keep well separately, but not after they have been combined. The final reagent is prepared just before it is to be used for the test, by mixing the reagents in the proportions of 25 cc. of A to 0.75 cc. of B.

Alcohol, 95 per cent.

A saturated solution of ammonium sulfate.

Standard solution of potassium permanganate. 0.7 cc. of 0.1 N potassium permanganate diluted with water to 50 cc. This must be renewed frequently from 0.1 N potassium permanganate which has been standardized in the usual manner.

A *cobaltous sulfate standard* may be used instead. This is prepared by dissolving exactly 2.16 grams of pure *anhydrous* cobaltous sulfate in water and diluting it to 100 cc. This solution will retain its color fairly well if preserved in the dark. It does, however, deteriorate, and does not exactly match the azobilirubin color.

Both standards are prepared to match in color a solution containing bilirubin in a concentration of 1:200,000 or 0.5 mg. per 100 cc.

Direct reaction. To 1 cc. of serum, in a small test tube, are added 2 drops of the diazo reagent. The contents of the tube are mixed thoroughly and the time observed. If there is a ***prompt direct*** reaction a bluish-violet color will appear at once and reach a maximum intensity in less than thirty seconds.

In the ***delayed direct reaction*** a reddish color appears only after one to fifteen minutes or more, gradually deepening and becoming more violet later.

In the ***biphasic reaction*** a reddish color appears at once, but changes to violet and becomes deeper only after a definite interval.

In order to facilitate differentiation of these reactions McNee and Keefer (13) recommend the adoption of certain modifications proposed by Lepehne (10). Lepehne found that caffeine-sodium salicylate tended to accelerate the development of color. McNee recommends that 0.25 cc. of serum be placed in each of three small tubes. To tube I is added 0.2 cc. of water, to tube II a small flake of caffeine-sodium salicylate and, after this has dissolved, 0.2 cc. of fresh diazo reagent. After the color has developed completely in this tube 0.2 cc. of diazo reagent is added to tube III. By comparing the color development in tube III with the fully developed color in tube II and the colorless tube I, the nature of the reaction can be determined with considerably greater accuracy than is possible with the usual technique.

The indirect reaction

In the original van den Bergh technique (2) alcohol was first added to the serum and the color was then developed by the addition of diazo reagent. In most sera which gave only an indirect reaction this order of procedure was found to remove the bilirubin almost completely from the protein precipitate. In sera which contained very large quantities of bilirubin, and especially those that gave a prompt direct action, the alcoholic protein precipitate retained considerable amounts of the dye. The test in these cases gave low values. More complete recovery could be secured by a second alcoholic extraction of the precipitate, but this proved a rather unsatisfactory expedient, and complicated the technique considerably. Thannhauser and Anderson (18) circumvented the difficulty in another manner. They pointed out that the incomplete extraction depended upon two factors: 1) the adsorption by proteins, 2) the insolubility of bilirubin in alcohol. Although bilirubin is relatively insoluble in alcohol, azobilirubin is quite soluble in this medium. They therefore suggested adding the diazo reagent before the alcohol. To further promote separation of the alcoholic azobilirubin from the precipitate they added, with the alcohol, saturated ammonium sulfate. With these modifications more nearly quantitative recovery of the bilirubin is secured.

In his original studies van den Bergh (2) employed pure bilirubin standards, although he proposed as a substitute a freshly prepared ethereal solu-

tion of ferric sulfocyanate. The high cost of the pigment makes bilirubin standards prohibitive. The ethereal ferric sulfocyanate standard proved impracticable because of the inconstancy caused by evaporation of the solvent. This led van den Bergh (13) to substitute a cobaltous sulfate solution. Although this is widely used, it has not proved entirely satisfactory because its color does not exactly match that of the azobilirubin and because it is not durable. Rhamy and Adams (15) have, therefore, substituted a standard potassium permanganate solution. Although this also deteriorates, it can be renewed frequently without difficulty in most clinical laboratories, where standardized permanganate solutions are regularly employed for other analytical purposes.

Procedure

To 2 cc. of serum in a centrifuge tube add 1 cc. of diazo reagent. After a minute or two introduce 5 cc. of 95 per cent alcohol and 2 cc. of a saturated solution of ammonium sulfate. Mix the contents of the tube and centrifuge. The supernatant fluid is compared with the standard permanganate or cobaltous sulfate solution in a colorimeter. If 2 cc. of serum are not available 1 cc. may be used with proportionately reduced quantities of all the other reagents.

Calculation. The result of the test may be expressed in terms of milligrams of bilirubin per 100 cc. of serum. It is, however, common usage to express it in terms of bilirubin units, each unit representing in theory a concentration equivalent to that of the standard, or 0.5 mg. per 100 cc. As the serum, in the analytical procedure, is diluted 5 times, the formula for the calculation of the bilirubin is:

$$\frac{5\,S}{U} = \text{units of bilirubin,}$$

or

$$\frac{2.5\,S}{U}\cdot \quad \text{milligrams of bilirubin in 100 cc. of serum.}$$

where S and U represent colorimetric readings of standard and unknown, respectively.

Normal values. The serum of normal individuals contains from 0.2 to 0.5 units (0.1 to 0.25 mg. per 100 cc.) of bilirubin. In rare sallow-complexioned people who were apparently normal McNee (12) found as much as 3 units (1.5 mg. per 100 cc.). In patients with obstructive jaundice and a prompt direct reaction bile can usually be found in the urine when the serum

contains more than 4 units (2.0 mg. per 100 cc.). In hemolytic jaundice, however, the urine may be free from bile and the serum may give only a delayed direct or no direct reaction when the bilirubin content of the serum, measured by the indirect method, is far greater, as much as 15 units (7.5 mg. per 100 cc.).

BIBLIOGRAPHY

1. Bergh, A. A. Hymans van den, and Muller, P.: Über eine direkte und eine indirekte Diazoreaktion auf Bilirubin. *Biochem. Z.*, 1916, **77**, 90.
2. Bergh, A. A. Hymans van den, and Snapper, J.: Die Farbstoffe des Blutserums. I. Eine quantitative Bestimmung des Bilirubins im Blutsesum. *D. Arch. klin. Med.*, 1913, **110**, 540.
3. Bernheim, A. R.: The icterus index (a quantitative estimation of bilirubinemia). An aid in diagnosis and prognosis. *J. Am. Med. Ass.*, 1924, **82**, 291.
4. Ehrlich, P.: Verhalten des Harns zu Sulfodiazobenzol. *Z. anal. Chem.*, 1883, **22**, 301.
5. Ehrlich, P.: Sulfodiazobenzol als Reagens auf Bilirubin. *Z. anal. Chem.*, 1884, **23**, 275.
6. Feigel, J., and Querner, E.: Biliruibnämie in ihren physiologische-chemischen Beziehungen mit besonderer Berücksichtigung der diagnostischen Bedeutung. *Z. ges. exp. Med.*, 1919, **9**, 153.
7. Greene, C. H., Snell, A. M., and Walters, W.: Diseases of the liver. I. A. survey of tests for hepatic function. *Arch. Int. Med.*, 1925, **36**, 248.
8. Harrop, G. A., Jr., and Barron, E. S. G.: The nature of the Van den Bergh reaction. *Trans. Ass. Am. Physicians*, 1929, **44**, 143.
9. Hymans van den Bergh, A. A.: La recherche de la bilirubine dans le plasma sanguin par la méthode de la réaction diazoique. Réaction prompte and réaction ralentie. *Press Méd.*, 1921, **29**, 441.
10. Lepehne, G.: Untersuchungen über Gallenfarbstoff im Blutserum des Menschen. *D. Arch. klin. Med.*, 1920, **132**, 96.
11. Mann, F. C.: Modified physiologic processes following total removal of the liver. *J. Am. Med. Ass.*, 1925, **85**, 1472.
12. McNee, J. W.: Critical review. Jaundice: A review of recent work. *Quart. J. Med.*, 1922–23, **16**, 390.
13. McNee, J. W., and Keefer, C. S.: The clinical value of the Van den Bergh reaction for bilirubin in blood: with notes on improvements in its technique. *Brit. Med. J.*, 1925, **2**, 52.
14. Meulengracht, E.: Die klinische Bedeutung der Untersuchung auf Gallenfarbstoff im Blutserum. *D. Arch. klin. Med.*, 1920, **132**, 285.
15. Rhamy, B. W., and Adams, P. H.: A new standard for the Van den Bergh test. *J. Lab. Clin Med.*, 1927–28, **13**, 87.
16. Pröscher, F.: Ueber Acetophenolazobilirubin. *Z. physiol. Chem.*, 1900, **29**, 411.
17. Stetten Dewitt: The surgical value of the estimation of the bile pigmentation (Icterus index) of the blood serum. *Ann. Surg.*, 1922, **76**, 191.
18. Thannhauser, J. S., and Andersen, E.: Methodik der quantitativen Bilirubinbestimmung im menschlichen Serum. Über die Ehrlich-Pröscher'sche Reaktion. *D. Arch. klin. Med.*, 1921, **137**, 179.

APPROXIMATE AIR ANALYSIS WITH A GLASS SYRINGE. Y. HENDERSON AND A. L. GREENBERG[3]

This method has been devised primarily to analyze, within 1 volume per cent, the oxygen-enriched air of pneumonia tents.

Apparatus

The only apparatus required is a graduated glass syringe of about 25 cc. capacity. The plunger must move without resistance. The needle is attached to the tip of the syringe by a short piece of rubber tubing which is kept closed by a spring clamp, except when air or absorbent solutions are being drawn into the syringe. It is convenient to have a small stand in which the syringe can be held with the tip upwards when gas volumes are measured.[4]

Reagents

10 per cent potassium hydroxide, for absorption of CO_2.

Hyposulfite solution, described on page 113, for absorption of oxygen. The solution is made immediately before using, and is at once covered with a layer of oil to prevent oxidation.

Procedure

The barrel and plunger of the syringe are dried, and are lubricated with a drop of paraffin oil, which is worked about so that it forms a thin film over the inner surface of the barrel.

The needle is connected with the syringe by the rubber tube, and the gas sample is drawn in. If a sample of air from a pneumonia tent is drawn, the needle is thrust through the curtain into the tent, and the plunger of the syringe is worked back and forth 2 or 3 times. The pinch clamp on the rubber tube is then closed.

To measure the gas sample, 1 or 2 cc. of water are drawn into the syringe, which is at once placed upright, with the needle end uppermost and the weight of the syringe resting on the handle of the plunger. As soon as the syringe has lost the heat imparted to it by the hand and come to room temperature, the volume of the sample is read. The reading should be

[3] Henderson, Y., and Greenberg, L. A.: Gas analysis with an all glass syringe for pneumonia tents. *J. Am. Med. Assn.*, 1931, **96**, 1475.

[4] Syringes for this analysis, and convenient clamps to hold them upright for the gas readings, can be obtained from Eimer and Amend, New York City.

taken with as little delay as possible, or absorption of CO_2 by the water in the syringe may become appreciable.

To absorb CO_2, 2 or 3 cc. of the potassium hydroxide solution are drawn into the syringe, the spring clamp on the rubber tube being closed as soon as the fluid is in the barrel. The solution is rolled about the walls of the syringe for about a minute to absorb the CO_2. The syringe is then placed upright again, with the weight resting on the plunger handle, and the volume of gas is read as before.

To absorb oxygen, most of the KOH solution is expelled, and 2 or 3 cc. of the hyposulfite solution are drawn into the syringe. Absorption is accomplished by rolling and shaking the solution about the walls of the syringe. The volume of residual gas is then read as after CO_2 absorption. To make sure that oxygen absorption was complete, the solution is again rolled about the walls of the syringe, and the volume reading is repeated.

When each gas volume is measured, the reading is taken at the upper edge of the meniscus in the syringe, where the solution meets the glass. It is not taken, as in most volumetric readings, at the bottom of the aqueous meniscus. With the deep red hyposulfite solution, it is impossible to see the bottom of the meniscus, and it is necessary to take the other readings by the same technique used when hyposulfite is present.

Calculation

$$\text{Per cent } CO_2 = 100 \times \frac{V_{H_2O} - V_{KOH}}{H_2O}$$

$$\text{Per cent } O_2 = 100 \times \frac{V_{KOH} - V_{hypo}}{V_{H_2O}}$$

V_{H_2O} = volume of gas sample measured over water; V_{KOH} = volume of gas measured over KOH solution after absorption of CO_2; V_{hypo} = volume of gas measured over hyposulfite solution after absorption of oxygen.

In some cases it is known that the CO_2 content is insignificant and only the O_2 content is desired. The absorption of CO_2 with KOH solution can then be dispensed with, and O_2 and CO_2 absorbed together with the alkaline hyposulfite as soon as the sample has been measured. Then the calculation becomes:

$$\text{Per cent } C_2 + CO_2 = 100 \times \frac{V_{H_2O} - V_{hypo}}{V_{H_2O}}$$

A PIPETTE FOR THE HANDLING OF WHOLE BLOOD SAMPLES, FOR USE WITH THE GASOMETRIC APPARATUS (GUEST[5])

The pipette[6] shown in figure 92 is made from a three-way stop-cock with 2 mm. capillary tubes. The holes of the cock should fit exactly the openings of the upper and lower delivery tubes. Bulbs are blown in the tubes as shown, and the lower bulb A is calibrated by weighing mercury *to contain* 1, 0.5, or 0.2 cc. from the bottom of the stop-cock to the tip of the tube; the upper bulb B is calibrated by weighing water *to deliver* 1 cc. from the bottom of the stop-cock to a mark above the bulb.

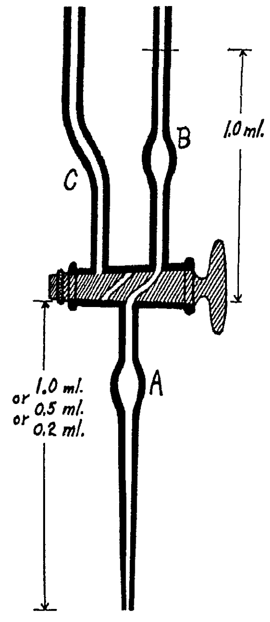


FIG. 92. Guest's automatic pipette for use in blood gas analyses. Bulb A is calibrated *to contain;* bulb B is calibrated *to deliver.*

To prepare the pipette for use in determination of CO_2 or O_2 in blood, CO_2-free water or air-free ferricyanide solution is drawn through A and B to the mark above B, care being taken that no air bubbles are caught at the capillary of the cock. The cock is then turned to deliver through the side arm C, and the bulb A and side arm C are dried by suction, alcohol and ether being used as usual.

[5] Guest, G. M.: A pipette for the handling of whole blood samples, for use with the Van Slyke gasometric apparatus. *J. Biol. Chem.*, 1931, **94**, 507.

[6] This pipette is made by the Central Scientific Company, Chicago.

The blood is drawn by gentle suction to slightly above the stop-cock in the side arm *C*. The cock is then turned at a right angle, automatically cutting off the exact sample in the bulb *A*, and a rubber band is then placed over the tip of the bulb *A* and around the hub of the stop-cock. The pipette may be carried thus to the laboratory (preferably in an upright position), or without a rubber band the pipette may be thrust into a test-tube filled with mercury, which effectively closes the lower end. The mercury may be chilled to stop enzyme changes in the blood.

For the delivery of the sample into the blood gas apparatus, the cup of the apparatus receives the usual amount of CO_2-free water or air-free ferricyanide solution, less 1 cc., and the tip of the pipette is inserted into the cup under this layer of fluid. The cock is then turned to deliver from the bulb *B*, and as the blood is delivered the water from bulb *B* rinses the blood from the lower bulb.

The tip of the pipette is provided with a rubber ring, as in figures 29 and 30.

DETERMINATION OF FATTY ACID UNSATURATION IN TERMS OF THE IODINE NUMBER.[7] PAGE, PASTERNAK, AND BURT[8]

Page, Pasternak and Burt[8] have slightly modified the Rosenmund-Kuhnhenn[9] method so that it is applicable as a micro-method to the various fractions (total lipoid, phosphatide, fatty acid) obtained in the Bang-Bloor method of blood lipoid analysis.

Reagents

Pyridine sulfate dibromide solution. Eight grams of pyridine and 10 grams of concentrated sulfuric acid are dissolved with ice cooling in 20 cc. each of glacial acetic acid. It is important to use only the purest acetic acid. The two solutions are now mixed together, and to the mixture 8 grams of bromine in 20 cc. glacial acetic acid are added. Making the volume up to 1 liter gives a 0.1 N solution. This is diluted as needed in the analysis.

Sodium thiosulfate, 0.1 N (see p. 33).

Potassium iodide, 10 per cent solution.

1 per cent starch solution, (see p. 34).

[7] The authors are indebted to Dr. I. H. Page for writing the description of this method.

[8] Page, I. H., Pasternak, L., and Burt, M. L.: Über den Transport von Fetten und Lipoiden durch Blut nach Oleingabe. *Biochem. Zeit.*, 1930, **223**, 445.

[9] Rosenmund, K. W., and Kuhnhenn, W.: Eine neue Methode zur Jodzahlbestimmung in Fetten und Olen unter Verwendung von Puridinsulfatdibromid. *Zeit. f. unter. d. Nahr. u. Genuss*, 1923, **46**, 154.

Procedure

About 3 milligram samples are employed, dissolved in 2 cc. of choloroform in a 50-cc. glass stoppered Erlenmeyer flask. One cubic centimeter of N/20 pyridine sulfate dibromide is added from a micro-pipette. The closed flask is allowed to stand exactly 15 minutes, 0.3 cc. of 10 per cent KI is added and the solution titrated to light yellow with N/20 sodium thiosulfate (see p. 13). Then add 2 or 3 drops of starch solution and titrate to the color of the blank. Towards the end of the titration it is important to shake the solution well. A blank is carried out at the same time to determine the titer of the pyridine sulfate dibromide solution. For certain oils such as linseed a slightly greater excess of the bromine solution is necessary.

Calculation

$$\text{Iodine number} = \frac{0.635 \times (A - B)}{\text{weight sample in grams}}$$

A = cc. N/20 sodium thiosulfate in blank.
B = cc. N/20 sodium thiosulfate in analysis.

The iodine number is the number of grams of iodine absorbed by 100 grams of lipoid.

The calculation of the number of molecules of iodine taken up per molecule of a fatty acid, and therefore of the number of unsaturated linkings in the fatty acid molecule, is made as follows.

$$\frac{\text{Moles } I_2}{\text{Moles fatty acid}} = \frac{\text{grams } I_2 \div 254}{\text{grams fatty acid} \div \text{molecular weight}}$$

$$= \frac{0.00635\ (A - B)}{\text{grams fatty acid}} \times \frac{\text{molecular weight of fatty acid}}{254}$$

SIMPLE TESTS FOR PLASMA PROTEIN CONTENTS BELOW THE EDEMA-PRODUCING LEVEL

As shown in the chapter of Volume I on the proteins of the body fluids, depletion of the plasma proteins produces a tendency to edema formation of the type ordinarily encountered in nephritis. The albumin appears to be more than twice as efficient, gram for gram, as is the globulin in maintaining the colloid osmotic pressure of the plasma and thereby preventing edema formation, so that the total protein content indicates the state of the blood, with respect to edema-forming tendency somewhat less exactly than it would be indicated by complete albumin and globulin determinations. Never-

theless, as shown by figure 70, page 683 of Volume I, the total protein content is in the great majority of nephritic cases closely related to the edema forming tendency: below a critical level of about 5.3 per cent of total protein, and a specific gravity of 1.023 in the plasma, edema is almost uniformly present. When protein analyses can not be done, it is frequently of interest to ascertain by a quick and simple test merely whether the protein content is markedly above or below this critical level. Two tests for this purpose will be described.

SPECIFIC GRAVITY TEST BY SUSPENDED DROP. PAGE AND VAN SLYKE[10]

Principle. The principle employed is the well known one of suspending a drop of plasma in a fluid of known specific gravity, and observing whether the drop rises or falls. In this case the fluid used is either monofluorobenzene or a mixture of monochlorobenzene and xylene. If kept in a closely stoppered bottle this mixture will remain unchanged for some months, perhaps indefinitely. Fluorobenzene has the advantage that it can not change by evaporation, but is somewhat more expensive than the xylene-chlorobenzene mixture.

Reagents

Xylene-chlorobenzene mixture. One volume of xylene is mixed with 2.06 volumes of monochlorobenzene.

Fluorobenzene, pure.

The specific gravity of either the mixture or the fluorobenzene should be 1.0235 at 20°. It should be tested. If an accurate pycnometer is not available, the following test may be applied. A salt solution of specific gravity, $D_{20°}^{20°} = 1.0235$, is prepared by dissolving 3.37 grams of pure fused NaCl in water and making up to 100 cc. A drop of solution, let fall as described below into the fluorobenzene or the xylene-chlorobenzene mixture, should remain suspended, and neither rise nor fall in the organic fluid.

Procedure. **The blood is drawn, mixed with heparin or not over 2 mg. of oxalate per cubic centimeter and centrifuged, as described on pages 689 or 61. A few cubic centimeters of the organic mixture are placed in a test tube. The temperature should be between 20° and 25°.**

A drop of the plasma is let fall into the mixture from a pipette, the tip of which is held 1 or 2 cm. above the surface of the organic fluid. If the plasma drop rises the specific gravity is below the critical level, 1.023. Plasma with specific gravity near the critical level will float about within the organic fluid, with little tendency to rise or fall.

[10] Page, I. H., and Van Slyke, D. D.: Unpublished.

COLORIMETRIC TEST OF FISHBERG AND DOLIN[11]

Principle. This test depends upon the buffer power of the plasma proteins. If a definite amount of standard acid is added to plasma, the acid is in part neutralized by the plasma bicarbonate. The rest of the acid reacts with the alkali proteinates of the plasma proteins, in the manner indicated by the equation, HCl + B Protein = BCl + H Protein. The less the amount of protein present, the greater will be the proportion of it changed by a given amount of HCl into the acid form, H Protein, and the lower will the pH of the acid-plasma mixture. If the plasma bicarbonate is constant, the fall in plasma pH caused by addition of a suitable constant proportion of HCl is a nearly constant linear function of the protein content. In Fishberg and Dolin's test, HCl equivalent to 50 millimoles per liter of plasma is added. The 25–30 millimoles of plasma bicarbonate normally present react with part of the HCl, leaving 20–25 millimoles to react with the proteinates. It is obvious that if the plasma bicarbonate is significantly above or below the normal range, the amount of HCl left to react with the protein will be too much altered to permit reliable results. When there is no alkalosis or acidosis, however, the plasma bicarbonate is within sufficiently constant limits to permit application of the method.

Reagents

0.1 N hydrochloric acid.

Methyl red solution, see table 68, page 812.

Procedure. To 0.5 cc. of serum add 0.5 cc. of distilled water, 0.25 cc. of 0.1 N hydrochloric acid, and 2 drops of methyl red solution. The relationship of color to protein content is indicated by the following table.

Color	*Per cent of protein*
Yellow	Over 6
Yellow-orange	About 5
Pink-orange	About 4
Pink	About 3
Red	Below 3

MANOMETRIC DETERMINATION OF FREE AMINO ACID NITROGEN IN URINE. VAN SLYKE AND KIRK[12]

The nitrous acid gasometric method is used (see p. 385). Ammonia and urea must be removed because both react with nitrous acid. The removal is accomplished, as in a former application of the nitrous acid reaction to

[11] Fishberg, Ella H., and Dolin, B. T.: Determination of serum proteins. *Proc. Soc. Exp. Biol. Med.*, 1930, **28**, 205.

[12] Van Slyke, D. D., and Kirk, Esben. Unpublished.

urine,[13] by changing the urea to ammonium carbonate with urease; the total ammonia being then removed. It has been found that distillation *in vacuo* is the most convenient and certain way to remove the ammonia. Boiling or concentrating at 100° with even mild alkalies results in some loss of amino nitrogen. Precipitation of ammonia with phosphotungstic acid is not sufficiently complete. Absorption with permutit, when used with the repetition necessary to remove the last traces of ammonia, proved more time-consuming and less certain that distillation *in vacuo*.

As shown previously,[13] urine contains amines other than the amino acids, which react slowly with nitrous acid. To correct for the relatively small amount of nitrogen from such amines which reacts during the 3 to 5 minutes required for complete reaction of the amino acid NH_2 groups, the reaction with nitrous acid is carried out in two periods. The first, of 3 to 5 minutes, depending on temperature (p. 395) gives the nitrogen of the amino acids plus a smaller amount from slowly reacting amines. The nitrogen yielded during this period is collected separately, and the reaction is continued for a second, equal period. The slowly reacting amines yield nearly the same amount of nitrogen during the second as during the first period. Consequently the amount obtained in the second period is subtracted from that of the first, in order to obtain the amino acid nitrogen.

Reagents

Urease, 10 per cent solution (see p. 545).

Phosphate buffer, 5 grams of KH_2PO_4 and 5 grams of $Na_2HPO_4 \cdot 12H_2O$ (or 2 grams of anhydrous Na_2HPO_4) per 100 cc.

Zinc sulfate 10 per cent. Ten grams of $ZnSO_4 \cdot 7H_2O$ per 100 cc. (see p. 481).

Sodium hydroxide, about 1 N.

Thymol blue, 0.1 per cent, see p. 812.

Reagents for manometric amino nitrogen on p. 386.

Two Hempel pipettes of the kind shown in figures 55–57, p. 388–90, are filled with alkaline permanganate.

Procedure

Digestion with urease. Ten cubic centimeters of urine are mixed in a 100 cc. volumetric flask with 0.5 cc. of 10 per cent urease solution, 10 cc. of phosphate buffer solution, and 50 cc. of water. The mixture is saturated with toluene and left at incubator temperature (35° to 40°) over night for the

[13] Van Slyke, D. D.: Improved methods in the gasometric determination of free and conjugated amino acid nitrogen in urine. *J. Biol. Chem.*, 1913, **16**, 125.

urease to complete the hydrolysis of the urea. Then 10 cc. of the zinc sulfate solution are added to precipitate proteins and clear the urine, and the mixture is diluted up to 100 cc. and filtered.

Distillation of ammonia. Fifty cubic centimeters of the filtrate are transferred to the 500 cc. double-necked distilling flask shown in figure 93, a drop of thymol blue is added, and enough sodium hydroxide, drop by drop, to turn the solution blue. The distilling and condensing flasks are connected by the familiar arrangement shown in figure 93, the system is evacuated, and the distilling flask is then immersed in a bath of warm water. A slight stream of air drawn through the fine capillary serves to maintain smooth boiling, but if the capillary is drawn properly fine, the volume of air let in

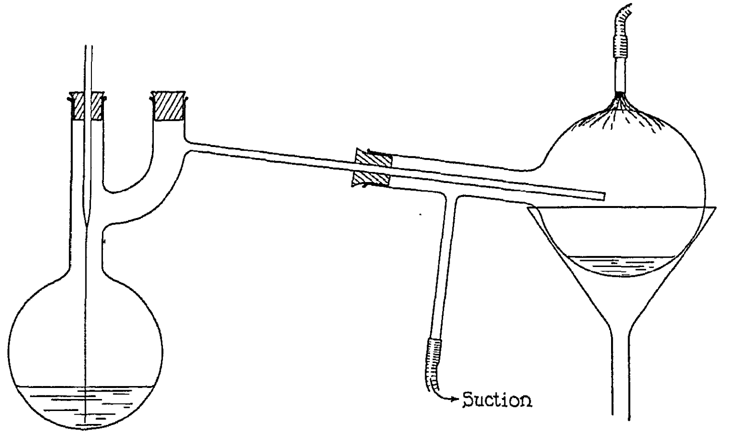


FIG. 93. Apparatus for rapid concentration of water solutions under diminished pressure

does not significantly affect the vacuum. The capillary may be replaced by pieces of porous clay. The rubber stoppers should be cleaned, as described in Chapter I, and moistened with water to make air-tight connections with the glass. With a properly assembled distilling apparatus, distillations can be run off at the rate of about one each 15 minutes, and require little attention. Distillation is continued until the volume of residual solution is diminished to about 10 cc. Then the solution is acidified with a few drops of glacial acetic acid, and submitted to vacuum for a moment to remove CO_2 bubbles.

The residue is washed with several small portions of water into a 25-cc. measuring flask, and brought to 25-cc. volume.

Determination of amino nitrogen. In a 5-cc. portion the amino nitrogen is

determined as described on pages 385–94. The duration of the reaction with nitrous acid is accurately set according to the temperature as shown in figures 58 on page 395. At the end of the reaction period the $NO\text{-}N_2$ gas mixture is run into a Hempel pipette provided with permanganate, as described on pages 390–91.

After the transfer, however, the nitrous acid-urine reaction mixture is not ejected from the manometric chamber. Instead, it is lowered in the chamber until the mercury is a little above the 50- cc. mark, and the reaction is permitted to continue again exactly as long as during the first reaction period. The duration of the second reaction is measured from the moment the first one was ended. During the last minute of the second reaction the chamber is shaken, as usual, and the gases are then transferred to the second permanganate-filled Hempel pipette.

The $N_2\text{-}NO$ mixtures in both Hempel pipettes are freed of NO as described on page 391. The residual N_2 from each is returned to the manometric chamber as described on page 391, and is measured as described on page 392. Both gas portions may be measured in succession over the same portion of deaerated water. Usually there is sufficient nitrogen formed during the first period to exert over 100 mm. pressure at 2 cc. volume: if the pressure at 2 cc. is less than 100 mm. better accuracy can be obtained by measuring at 0.5 cc. volume.

Blank analyses are carried through in the same manner, including urease digestion and zinc treatment, except that water replaces the urine.

Calculation

$$P_{N_2}\ (\textit{first period}) = p_1 - p_0 - c_1$$

$$P_{N_2}\ (\textit{second period}) = p_2 - p_0 - c_2$$

$$P_{N_2}\ (\textit{amino acid}) = P_{N_2}\ (\textit{first period}) - P_{N_2}\ (\textit{second period})$$

In these formulae, p_1 and p_0 have the significance indicated under "Measuring nitrogen gas" on page 392, p_2 is the reading corresponding to p_1 but obtained during the second reaction period, c_1 and c_2 are the values of P_{N_2} for the first and second reaction periods respectively, obtained in the blank analyses.

To calculate grams of amino acid nitrogen per liter of urine, P_{N_2} (*amino acid*) is multiplied by the proper factor from the second or third column of table 40 on page 399. Since the sample represents 1 cc. of urine, the amino acid nitrogen found in it represents milligrams in 1 cc. of urine, or grams in a liter.

CHLORIDE IN BLOOD PLASMA. OPEN CARIUS DIGESTION WITH IODOMETRIC TITRATION. VAN SLYKE, ALVING, AND HILLER[14]

Plasma or serum is digested with nitric acid and silver nitrate, and the silver chloride is filtered off. In aliquots of the filtrate the excess silver is titrated iodometrically by the method of McLean and Van Slyke (p. 832).

One cubic centimeter of plasma suffices for duplicate analyses. The method, in the writers' hands, has offered the nearest approach to micro quantities without loss of accuracy. For discussion of the open Carius digestion and of the iodometric titration, see p. 831, 832, 845.

Reagents

The 0.05 N silver nitrate in concentrated nitric acid, or the alternative 0.15 N aqueous silver nitrate, described on pages 835 and 836.

Buffered starch-nitrite-citrate soltion, see p. 844.

0.01 N potassium iodide. To standardize the iodide, 3.000 cc. of the 0.05 N $AgNO_3$ in concentrated HNO_3 are diluted to 25 cc., and 10 cc. aliquots are treated with starch-nitrite solution and titrated with the iodide as described below. Of the 0.01 N iodide, the amount required should be 6.00 cc. plus the amount required to give the end-point, which is usually 0.15 cc. (see p. 845).

Procedure

One cubic centimeter of plasma is digested in a 25-cc. Pyrex volumetric flask as described on p. 836–7, with 3 cc. of the acid 0.05 N silver nitrate, or with 1 cc. of 0.15 N aqueous silver nitrate plus 3 cc. of concentrated nitric acid. The digested mixture is cooled, diluted to the mark, and filtred through a dry paper.

Of the filtrate 10 cc. are pipetted into a 50 cc. flask, or wide test tube. Four cubic centimeters of the starch-nitrite solution are added, and the mixture is titrated to a blue end-point with 0.01 N iodide from a micro burette.

To assist in detecting the end-point, a control is prepared as follows: One cubic centimeter of the 0.05 N silver nitrate in concentrated nitric acid is placed in a 25-cc. flask, together with 2 cc. of additional concentrated HNO_3. The mixture is diluted to 25 cc. A 10-cc. portion is treated with with 4 cc. of starch-nitrite solution, and is titrated past the end-point with the 0.01 N postassium iodide. Then a drop or two of the 0.05 N silver nitrate is added to remove the blue color. This tube, containing a fluid of turbidity

[14] Van Slyke, D. D., Alving, A., and Hiller, A. E.: Unpublished.

similar to that of the titrated tube, is held near the latter during titration. When as much as 0.01 cc. of excess 0.01 N KI is added to the latter, it becomes obviously darker than the control. The difference is most readily seen by looking down through both tubes upon a white surface below. See remarks concerning end-point on page 845.

Calculation

$$25\,(B - A) = \text{milli-equivalents of Cl per liter plasma}$$
$$146.2\,(B - A) = \text{chloride calculated as milligrams NaCl per 100 cc.}$$

B = cc. of 0.01 N iodide to titrate a blank. The value of B is approximately 6.15 cc. A = cc. of iodide required in the analysis.

ANALYSIS OF GASTRIC CONTENTS FOR FREE HYDROCHLORIC ACID, TOTAL CHLORIDES, AND TOTAL BASE

Clearing gastric contents

The contents may be *filtered through a folded paper* if there is sufficient time and material, but the filtration is usually slow.

Centrifugation removes the solid particles, but not the mucous, which rises towards the top. Centrifugation followed by filtration may be satisfactory.

As a general method, G. A. Harrison especially recommends *filtration by pressure through closely packed absorbent cotton.*[15] He states that each of the following procedures for this purpose is easily carried out, but that the preferable one from the point of convenience is that in which centrifugation is used. He attributes it to the school of Sir F. Gowland Hopkins. The sample of gastric contents is placed in a heavy centrifuge tube with straight, non-tapering walls. A wad of absorbent cotton about an inch thick is tightly packed together and forced into the tube. The latter is then centrifuged at high speed for 15 minutes. The cotton plug is forced into the bottom of the tube leaving a layer of clear gastric juice above.

Instead of centrifuging, the cotton plug may be pushed down through the fluid with a very stout footed glass rod.

Another alternative employs a syringe in place of a centrifuge tube. The cotton plug is pressed into the tip of the barrel, the gastric fluid is then poured in. The plunger, lubricated with paraffin oil, is then inserted into the barrel, and is pressed to force the fluid through the cotton.

[15] Harrison, G. A.: Chemical Methods in Clinical Medicine. London, 1930.

Titration of free hydrochloric acid

The gastric contents are titrated with alkali to an end-point of approximately pH 2 with thymol blue as indicator. At this point free HCl in 0.01 N concentration remains still untitrated. This is equivalent to 10 cc. of free 0.1 N hydrochloric acid per 100 cc. of gastric contents. To be exact, therefore, one should add 10 millimoles per liter, or 10 cc. of 0.1 N HCl per 100 cc., to the result obtained by the titration. The more common procedure is to neglect this addition, and to express as free HCl only that titrated to the end-point of thymol blue or an indicator of similar acid end-point.

Reagents

0.1 N sodium hydroxide, see page 31.
0.1 per cent thymol blue solution, see table 68, page 812.

Procedure

To 10 cc. of the cleared fluid 4 or 5 drops of thymol blue solution are added. The 0.1 N sodium hydroxide is then added from a burette until the red changes to a yellow orange.

Or a micro titration may be performed on 2 cc. of the cleared contents, to which 1 drop of thymol blue solution is added. In this case the 0.1 N alkali is added from a micro burette, such as that of Bang (figure 1, page 13).

Calculation

The results, expressed as either cc. of 0.1 N free HCl per 100 cc. of gastric contents, or as millimoles of HCl per liter of contents, are calculated by the formula,

$$\frac{100 \times (\text{cubic centimeters of } 0.1\ N \text{ NaOH used in titration})}{\text{cubic centimeters of contents titrated}}$$

When the sample is 10 cc., the result is therefore calculated by multiplying the cubic centimeters of 0.1 N alkali used by 10, while when the sample is 2 cc. the cubic centimeters of alkali are multiplied by 50.

A correction of 10 for untitrated acid may be added to the result, as explained above.

Remarks

It has been customary to perform a second titration from the end-point of the above titration to an alkaline end-point of pH about 8.5 with the end-point of phenolphthalein or of the yellow-blue alkaline change of thymol blue. The alkali used to titrate from pH 2 to 8.5 has been expressed as a

measure of the organic acids, or as the rest of the total acidity of the gastric fluid. Such a titration would in fact include the organic acids (see page 646), but it is uncertain what other buffers, basic and acid, active between pH 2 and 8, it also includes in gastric fluid collected under various conditions. (See discussion of buffers, page 888 of Volume I.) Unless a well defined especial object is in view, there seems at present to be little advantage in performing this added titration: the interpretation of the results is too indefinite.

Determination of total chlorides

On a sample of 1 cc. or less of cleared contents, the chloride may be determined by the open Carius method described for blood serum on pages 835–838.

Determination of total base

The methods for total base in blood serum described in chapter 23 may be applied to gastric contents.

In acid gastric juice, the difference, Cl − total base, represents approximately the free HCl.[16]

SULFATE IN BLOOD. MICRO TITRATION METHOD OF COPE[17]

The benzidine sulfate precipitates obtained in Hubbard's methods, (p. 902–905) are titrated with 0.02 N alkali, according to the principles outlined on page 890, by means of Rehberg's micro burette. Cope was able thus to obtain more reliable results than by colorimetric estimation of the benzidine in the precipitates.

Apparatus

Rehberg's micro burette, described on page 14.

Conical Pyrex centrifuge tubes.

Glass steam jacket, shown in figure 93, for heating the bottom of the centrifuge tube during titration.

Reagents

Benzidine base, 1 per cent solution in pure acetone.

Trichloroacetic acid, 20 per cent, sulfate-free (see page 899).

Pure acetone.

0.02 N sodium hydroxide, carbonate-free (see p. 29).

[16] See Gamble's work reviewed on p. 773–776 of Volume I.

[17] Cope, C. L.: Determination of inorganic sulfate in human blood plasma by microtitration. *Biochem. J.*, 1931, **25**, 1183.

Procedure

The serum filtrate and the precipitates of benzidine sulfate are prepared and washed as described on pages 903 and 904. The same amounts of serum filtrate are taken, so that each portion represents 1 cc. of serum.

The washed precipitates are titrated as follows: To the precipitate in the tube 0.5 cc. of water is added. Phenolphthalein is added by dipping the tip of a fine glass rod into the indicator solution and inserting it into the centrifuge tube, where it may be allowed to remain, as it is useful in breaking up the precipitate during the titration. The centrifuge tube with its fine glass

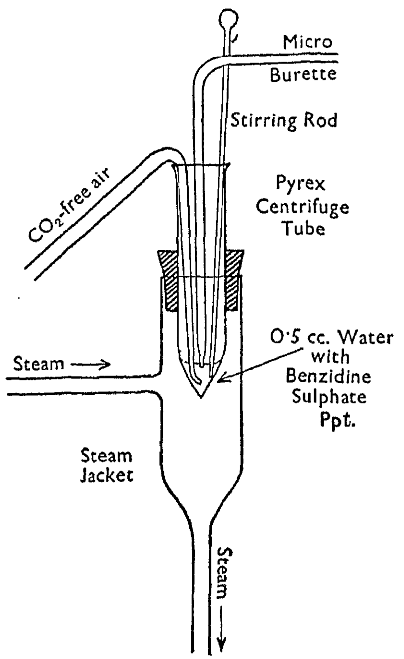


Fig. 94. Glass steam jacket for heating tube in which micro titration of benzidine sulfate is done. From Cope.[15]

stirring rod is then inserted into the steam jacket, as shown in figure 93, and the whole is arranged so that the tip of the micro burette just tips beneath the surface of the liquid in the tube. The tube for bubbling CO_2-free air is next placed in position, and a gentle stream is commenced.

It is advisable to carry the titration as far as possible before the steam is turned on, in order to avoid heating the burette more than is necessary.

When the pink color due to the added alkali tends to become persistent, the steam is turned on, and the titration is completed as rapidly as possible at 100°. No difficulty is encountered in detecting the first appearance of a permanent pink if good lighting be arranged. Before removing the tube its sides are well washed down by turning on the air supply fully for a moment, and further alkali is added if necessary. The centrifuge tube and jacket are then immediately removed from the tip of the burette. Some haste is desirable during these later stages in order to minimize the heating of the fluid in the burette during the titration. After the tube is withdrawn cooling leads to a slight retraction of fluid from the tip of the burette. The mensicus must be brought once again to the tip by turning the control screw, before the final reading of the burette is made.

A blank titration is necessary in order to determine the amount of alkali required to produce a definite pink color in 0.5 cc. of distilled water at 100°. This usually lies between 3.0 and 4.5 cubic millimeters of 0.02 N sodium hydroxide, and the blank must be subtracted from the titration figure.

Calculation

The calculations are the same given on pages 896–897 for the benzidine titration method applied to urine, since 0.02 N alkali is used both for that and this procedure. In the present blood analyses, the volume V is 1 cc. One additional formula may be needed for blood:

$$32\ (A - C) = \text{milligrams of sulfur per 100 cc. of serum}$$

SIMPLIFIED COLORIMETRIC DETERMINATION OF BLOOD UREA CLEARANCE. VAN SLYKE AND COPE[18]

Principle. The urea contents of urine and blood are compared in a colorimeter in such a manner that a single reading gives directly the percentage of average normal renal function in terms of the clearance. The urine is first diluted to such an extent that if the clearance, either standard or maximum, is the average for a normal subject, the urea concentrations in blood and diluted urine will be equal. The urea in both blood and urine is converted into ammonia with urease, proteins and other interfering substances are removed, and the ammonia contents of the two filtrates are compared colorimetrically.

[18] Van Slyke, D. D., and Cope, C. L.: Simplified colorimetric determination of blood urea clearance. *Proc. Soc. Exp. Biol. Med.*, May, 1932.

For a discussion of the blood urea clearance, see page 564, this volume, and page 345 of Volume I.

No standard solutions are required, because the blood is compared directly with the urine.

The number of times the urine must be diluted for comparison with the blood is found by reference to the curve of figure 95.

This curve is computed as follows: The average *maximum* clearance (for urine volumes over 2 cc. per minute) is 75 (see p. 564–65). The maximum clearance is calculated as

$$C_m = \frac{U\ V}{B}$$

where V is reckoned in cubic centimeters of urine per minute, B is blood urea concentration, and U is urine urea concentration. When C_m has the average normal value of 75, the ratio U/B is calculated therefore as $U/B = 75/V$. When V is expressed in cubic centimeters of urine per hour, the calculation changes to $U/B = 4500/V$. The ratio U/B is the number of times a portion of the urine must be diluted in order to bring its urea concentration down to that of the blood, when the clearance is normal. The U/B values thus calculated are expressed by the higher part of the curve of figure 95.

If the urine volume is below 2 cc. per minute, the formula which holds is that of the standard clearance, $C_s = \frac{U\sqrt{V}}{B}$ and the average normal value of C_s is 54. The number of times dilution required for urines excreted at rates less than 2 cc. per minute is therefore calculated as $U/B = 54/\sqrt{V}$ when V is expressed in cubic centimeters of urine per minute, or as $U/B = 54 \times \sqrt{60}/\sqrt{V} = 418/\sqrt{V}$ when V is expressed in cubic centimeters of urine per hour. The dilution ratios thus calculated are indicated by the lower part of the curve of figure 95. Plotting the curve logarithmically gives it a rectilinear form, with a break at the augmentation limit where the C_s formula is replaced by the C_m formula.

Reagents

Permutit, see p. 577–78.

Urease, 10 per cent solution, see p. 545.

Phosphate buffer, 5 grams of KH_2PO_4, and either 5 grams of $Na_2HPO_4 \cdot 12H_2O$ or 2 grams of anhydrous Na_2HPO_4, per 100 cc.

Somogy's acid zinc sulfate solution for precipitating proteins. 12.5 grams of $ZnSO_4 \cdot 7H_2O$ and 125 cc. of 0.25 N sulfuric acid diluted to 1 liter.

0.75 N sodium hydroxide.

Nessler's solution, p. 532.

Sodium citrate, pulverized.

Conditions for collecting blood and urine

These are outlined on page 564, where there is also a general discussion of the clearance. Urine is collected during two successive periods of one hour each, and the volume in each hour is accurately measured. Blood is drawn at about the middle of the two-hour period. *Citrate instead of oxalate* should be used to prevent coagulation. Oxalate is likely to cause development of turbidity when the blood filtrate is nesslerized.

Treatment of urine

1. *Removal of preformed ammonia.* Of the urine collected during each hour, place about 10 cc. in a small Erlenmeyer flask with 2 grams of permutit

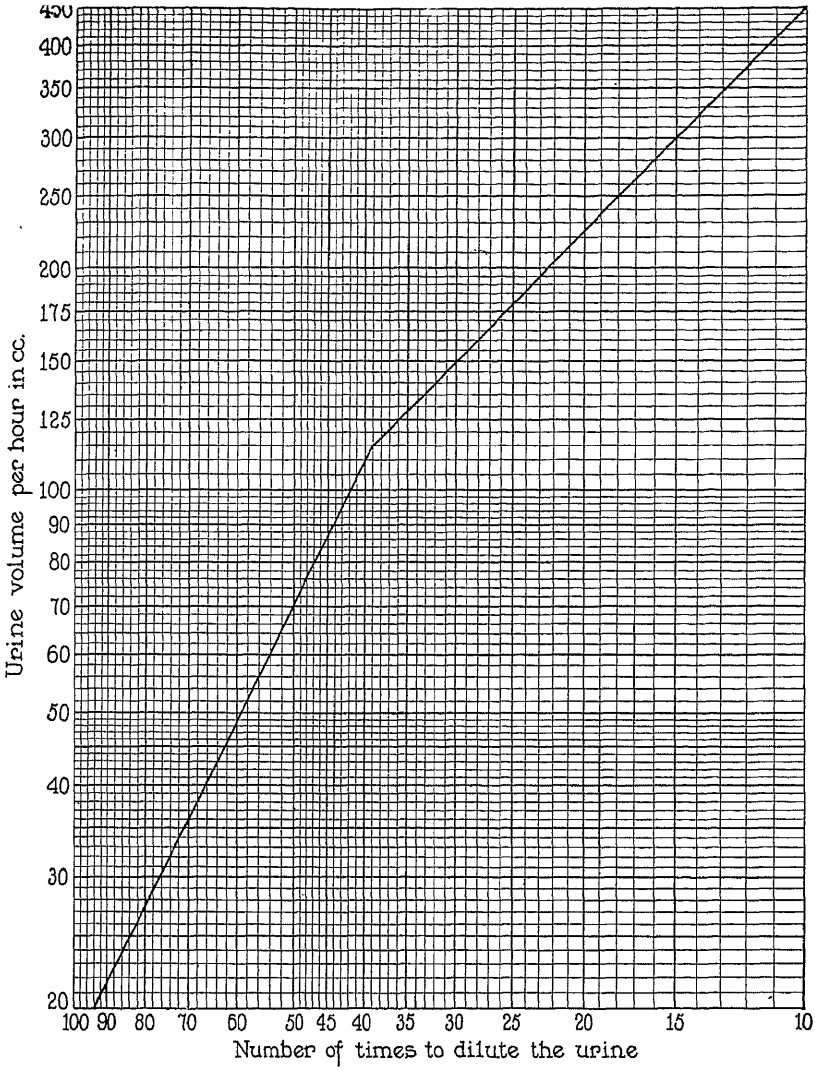


FIG. 95. Urine dilution chart for simplified blood urea clearance

and rotate for 5 minutes to absorb ammonia. Filter through a dry filter paper.

2. *Treatment with urease.* Place in a 100-cc. cylinder a volume of permutit-treated urine, 1, 2, or more cubic centimeters, such that when later diluted according to figure 95 the resultant volume will be between 50 and 100 cc. To the undiluted urine in the cylinder add 5 cc. of the phosphate buffer solution and 1 cc. of the 10 per cent urease. Let stand 10 minutes, preferably not much longer in order not to give ammonia opportunity to volatilize.

3. *Dilution and removal of protein and other interfering substances.* After the urease has acted 10 minutes 3 cc. of the zinc sulfate solution are added. It reacts with the phosphate buffer to produce a precipitate which clears the urine. The mixture is then diluted up to the volume calculated from figure 95. After standing a few minutes, the solution is passed through a dry filter. A slight cloudiness may be present in the filtrate, but the turbidity disappears when the filtrate is nesslerized. A portion of the filtrate, 2 or 5 cc., is pipetted into a volumetric flask and diluted 10 times, in order to parallel the 10-fold dilution which the blood undergoes.

If the subject is a child, or deviates greatly from usual adult size, a correction for body size is introduced by multiplying the observed volume of the hour's urine by the proper factor from figure 78, p. 569, and using the urine volume thus multiplied as the ordinate for figure 95.

Example. If the urine volume per hour is 80 cc., figure 95 indicates that the urine should be diluted 46.8 times. This holds for any adult within usual size limits.

If, however, the subject is a child of 1.40 meters height, the 80 cc. observed hour's volume is multiplied by the factor 1.49 from figure 78. The corrected volume, 119 cc., is then interpolated on the curve of figure 95, and indicates that the urine should be diluted only 37.5 times.

Treatment of blood

1. *Digestion with urease.* The volume of blood sample required depends upon the volume of solution necessary for a reading in the colorimeter used. If 5 cc. of solution suffice for the colorimeter, 1 cc. of blood will do, unless an unexpectedly low clearance value necessitates the use of a fresh portion of of filtrate for supplementary dilution. In general, a 2-cc. sample is adequate.

The sample is mixed with 0.1 its volume of 10 per cent urease, and let stand 10 or 15 minutes.

2. *Removal of blood proteins.* For each cubic centimeter of blood sample, 7.9. cc.of Somogyi's acid zinc sulfate solution are added, then 1 cc. of 0.75 N sodium hydroxide. After standing for some minutes the fluid is passed through a dry filter.

Nesslerization and comparison of blood and urine filtrates

Equal volumes of the blood filtrate, 5, 10, or 20 cc., as required by the colorimeter, and of the 10-fold diluted urine filtrate are pipetted into dry vessels, and a trace of sodium citrate powder is added from the point of a knife to each and allowed to dissolve. The citrate retards development of turbidity after nesslerization. To each measured portion of filtrate one-tenth its volume of Nessler's solution is added, and the solutions are at once compared in a colorimeter. The nesslerization may be done in the colorimeter cups if they are dried before the filtrates are measured in.

Calculation

If the colors of the two filtrates are such that the higher scale reading does not exceed twice the lower, the clearance is calculated directly from the colorimeter scale readings.

$$\text{Per cent of average normal clearance} = \frac{100\ (B)}{(U)}$$

(B) represents the reading of the blood filtrate, and (U) the reading of the urine filtrate. (The readings are inversely proportional to the concentrations, hence the inversion of the usual concentration formula.)

If the clearance is less than half average normal, the blood filtrate when nesslerized will be more than twice as deep in color as the urine filtrate. In such a case the preliminary $(B)/(U)$ reading is made in the above manner. Then a fresh portion of the blood filtrate is diluted 2, 3, 5 or 10 times, as indicated by the preliminary reading, in order to bring the $(B)/(U)$ ratio into the neighborhood of unity. A portion of the diluted blood filtrate is then nesslerized and compared with a fresh portion of the urine filtrate simultaneously nesslerized. The calculation then becomes:

$$\text{Per cent of average normal clearance} = \frac{100\ (B)}{D\ (U)}$$

D represents the number of times the blood filtrate has been diluted, before the final nesslerization in cases where a low clearance necessitates such dilution. If the subject is known to have a low clearance, the time required for the preliminary reading can usually be saved by diluting the blood filtrate to the extent probably necessary before the first reading is made.

Clearances by this method are not so accurate as those based on gasometric or titrimetric urea determinations, from which they may deviate by several parts per hundred. The accuracy appears, however, to suffice for most of the purposes to which the clearance is applied.

1931

INTERNATIONAL ATOMIC WEIGHTS

NAME	SYMBOL	ATOMIC WEIGHT
Aluminum	Al	26.97
Antimony	Sb	121.26
Arsenic	As	74.93
Barium	Ba	137.36
Bismuth	Bi	209.00
Boron	B	10.82
Bromine	Br	79.916
Calcium	Ca	40.08
Carbon	C	12.00
Chlorine	Cl	35.457
Chromium	Cr	52.01
Cobalt	Co	58.94
Copper	Cu	63.57
Fluorine	F	19.00
Gold	Au	197.2
Hydrogen	H	1.0078
Iodine	I	126.932
Iron	Fe	55.84
Lead	Pb	207.22
Magnesium	Mg	24.32
Manganese	Mn	54.93
Mercury	Hg	200.61
Molybdenum	Mo	96.0
Nickel	Ni	58.69
Nitrogen	N	14.008
Oxygen	O	16.0000
Palladium	Pd	106.7
Phosphorus	P	31.02
Platinum	Pt	195.23
Potassium	K	39.10
Radium	Ra	225.97
Silicon	Si	28.06
Silver	Ag	107.880
Sodium	Na	22.997
Strontium	Sr	87.63
Sulfur	S	32.06
Tin	Sn	118.70
Tungsten	W	184.0
Uranium	U	238.14
Zinc	Zn	65.38

AUTHOR INDEX

H

K

SUBJECT INDEX

D

E

R